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Published quarterly at 600 S. Michigan Ave., Chicago, Ill., by the American Veterinary Medical Association. Entered as Second-Class Matter January 31, 1941, at the Post Office at Chicago, Illinois, under the Act of March 3, 1879. Subscription price: $4.00 per year for United States, Canada, and countries affiliated with the Pan American Postal Union; foreign countries, $4.30. Members of the American Veterinary Medical Association may subscribe to the American Journal of Veterinary Research for $2.50 per year added to the annual dues of $7.00. Single copies $1.25. Address all correspondence to American Veterinary Medical Association.

American Journal of Veterinary Research

VOL. IV JANUARY, 1943 NO. 10

Sulfaguanidine in the Treatment of Infectious Enteritis in Swine

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St. Paul, Minnesota

THE USE OF sulfaguanidine as a chemotherapeutic agent in intestinal infections of man and animals is receiving much attention at the present time. Within recent months, reports[1-11] have appeared in medical and veterinary literature to indicate that this drug has a definite value in the treatment of some forms of intestinal diseases.

Sulfaguanidine, like some of the other sulfonamides, possesses bacteriostatic and bactericidal properties, particularly for the gram-negative types. The beneficial results of this drug in infections of the gastrointestinal canal are due in the main to the fact that it is not readily absorbed, so that relatively high concentrations can be maintained in the gut without developing sufficiently high blood concentrations to manifest serious toxic reactions. Thus, the bacteriostatic and bactericidal effects are exerted upon the primary cause at the point of greatest impact, the intestinal mucosa.

Our interest in the possibilities of sulfaguanidine as a therapeutic agent in the enteric infections of swine stems from the report of Marshall, *et al.*, in which they describe the results of an investigation on its use in the treatment of acute bacillary dysentery in children.

The enteric syndrome involved in this study has been described many times and under many different names; *i. e.*, enteritis, necrotic enteritis, diphtheritic enteritis, infectious enteritis, bacterial enteritis, salmonellosis, necrobacillosis, "necro," scours, bloody diarrhea and a number of other terms.

The etiology is not yet completely understood, although it is believed that organisms of the Salmonella group are the ones most commonly present. A limited number of cases was studied bacteriologically, and in every instance organisms of this group were isolated from the stool, submucosa of the colon, and in some cases from the mesenteric and sublumbar lymph nodes. *Salmonella choleraesuis* was found most often.

EXPERIMENTAL OBSERVATIONS

The clinical condition of the animals studied varied from acute, bloody, or hemorrhagic types to chronic forms of the disease. The swine included in this study all contracted and developed the disease under natural field conditions. The smallest herd from which pigs were obtained numbered 27; the largest, approximately 1,100. The percentage of animals affected in the different herds varied from 25 to over 80. The ages of the pigs affected ranged from approximately 3 weeks to 2 years, and the weights from 8 to 415 lb. Before any pigs in any of the herds were used for study, at least two and sometimes more of the clinically sick pigs were killed for postmortem examination, to assure the existence of an enteric involvement.

For convenience and clarity of presentation, this report is divided into four parts. The first deals with a series of controlled experiments on the use and value of sulfaguanidine from the standpoint of the therapy of enteric infections. The second

Paper No. 2015, scientific journal series, Minnesota Agricultural Experiment Station, University of Minnesota.
From the Division of Veterinary Medicine, University Farm.

concerns a series of cases treated under field conditions. It was not convenient in most instances, and impossible in others, to allow some of the affected pigs to be left untreated as controls. The progress of the disease was followed each day by trained observers in many of these cases, although in others the observations were made only periodically. Part 3 pertains to a study of the blood, fecal, and urine concentrations of the drug when administered at different dose levels for various lengths of time. The matters of drug tolerance and toxicity are discussed in part 4.

Throughout this report, the amount of drug administered is given in grams per 10 pounds of body weight per day (Gm./10 lb.). In all cases, except when otherwise noted, the initial dose was double the amount of the usual dose. The route of administration in all cases was by mouth, but the mode of administration varied, depending on the physical status of the pigs treated and, to some extent, on local conditions. The drug was put up in gelatin capsules and administered with a balling gun in some instances, suspended in water or milk and given as a drench or introduced *via* a stomach tube in others, or in certain other cases it was mixed with small amounts of mash or thick swill so that it would be consumed at feeding time.

Part I

Twelve groups or lots totaling 83 pigs were used in these experiments.

Group 1 (tables 1 and 2) contained only 2 pigs. These were the first to be treated and studied at this station. In May, 1941, 4 young pigs were brought to the laboratory for diagnosis. All were emaciated, moderately dehydrated, semiactive and manifesting a profuse, watery diarrhea. According to the owner, these cases were representative of a condition that involved several more pigs in his herd, and they had been sick for a week or more prior to this date. Two of the group were destroyed and careful autopsies held. Both carcasses showed marked diphtheritic and necrotic inflammatory changes that involved the mucosa of the cecum and colon. The remaining 2 were identified, weighed, and placed in a pen for treatment and observation. Pig 851 received 0.9 Gm./10 lb. of sulfaguanidine divided between morning and evening. The drug was put in gelatin capsules and administered with a balling gun. Pig 850 was left as the control. The stools of 851 were less watery on the fourth day of treatment, and in another four days they were formed and appeared normal. The physical condition of this pig began to improve on the fifth day and continued to improve thereafter. It was released as recovered on the twelfth day. The control continued to be sick. It lost 7 lb. in twelve days (weighing 33% less than when admitted). At this time it was placed on treatment, receiving the drug at the rate of 1.84 Gm./10 lb. (group 3). After four days, its stools were formed and continued as such for the following six days, at which time it was released as normal. The physical condition of this pig at the time of release was vastly improved over its condition at time of admission, and it gave evidence that with good care and diet it should grow and develop into a marketable hog.

Group 2 (tables 1 and 2) is a series of pigs of nursing age that averaged 12.1 lb. They came from a large herd where 50 per cent or more of similar ages were affected with an enteric disease. It was reported that the malady had been present in the herd for about two weeks and that between 40 and 60 pigs had died during the past several days. We obtained 20 of the sick pigs for study, but before their arrival 6 of the group had died. Postmortem examination of each of the 6 dead pigs revealed lesions characteristic of enteritis. Seven of the lot were put on treatment and 7 left as controls. The daily dose varied for the different pigs from 0.82 Gm./10 lb. to 1.62 Gm./10 lb. and the mode of administration was by capsule. All treated cases recovered, as did 2 of the controls. Five of the controls succumbed before the treated pigs were released.

Group 3 contained 4 pigs. Two were treated and 2 served as controls. Of those treated, 1 received the drug at the rate of 1.10 Gm./10 lb. and the other at 1.84

TABLE I.—Treatment of Infectious Enteritis with Sulfaguanidine.

Group	Identification	Sex	Body Weight lb.	Daily Dosage* Gm./10 lb.	Days on Treatment and Character of Feces											
					1	2	3	4	5	6	7	8	9	10	11	12
1	850	M	20	0	vt	vt	vt	vt	vt		vt	vt	vt	vt	vt	vt†
	851	M	22	0 90	vt	vt	vt	s	s		s	f	f	f	f	R
2	859	F	14	0	vt	vt	D‡									
	860	M	15	0	vt	vt	vt	D ‡								
	861	F	14	0	vt	D‡										
	862	M	11	0	vt		vt	vt						f	f	R
	863	M	10	0	vt			vt		vt		vt	vt		vt	D‡
	864	F	11	0	vt		vt			vt			f	f	f	R
	865	M	9	0	vt	D‡										
	852	M	16	0.82	vt		vt			f			f	f		R
	854	M	15	0.86	vt		vt			f					f	R
	855	F	12	0.86	vt		vt						f		f	R
	853	F	13	1.00	vt		vt			f			f		f	R
	856	F	12	1.08	vt					s		s	f		f	R
	857	M	10	1.30	vt					vt					f	R
	858	M	8	1.62	vt		vt	vt	s			s		f	f	R
4	13–2	F	25	0	vt	vt	vt	vt	vt	vt	vt	vt	vt†			
	13–1	F	21	0.94	vt	vt	s	s	s	f	f	f	R			
	7–1	M	56	0.68	vt	vt	s	s	s	f	f	f	R			
10	3	M	12	0	vt		D‡									
	6	F	16	0		vt	vt		vt	s	vt	vt	D‡			
	9	F	20	0		vt	vt			vt			vt	vt		vt§
	11	M	14	0		vt	vt	D‡								
	4	M	12	1.0	vt	vt					vt	D‡				
	10	F	11	1.0	vt		D‡									
	13	M	20	1.0	vt		vt	vt		vt		s	s	f		f||
	14	M	19	1.0	vt		vt			vt	D‡					
	15	F	24	1 0	vt		D‡									
11	3	M	39	0	vtb		vtb		vt	vt	vt		D‡			
	7	M	24	0	vtb				vtb	vtb			vtb		vtb	vt§
	8	F	20	0	vtb	D‡										
	13	F	27	0	vtb	D‡										
	1	F	35	1.0	vtb	vtb			f	f	f			R		
	4	M	43	1.0	vt	vtb				f	f			R		
	9	F	39	1.0	vtb	D‡										
	11	M	35	1.0	vt	vt			s	f	f		f	R		
	15	M	21	1.0	vtb	vtb		vt		f	f		f	R		

b = bloody; t = thin; vt = very thin; s = soft; f = formed; D = died; R = released as recovered.
* = daily dose divided, morning and evening; † = later treated and recovered; ‡ = postmortem, infectious enteritis; § = very weak, dehydrated 20th day; || = died 23d day, inanition and cachexia.

Gm./10 lb. When they were placed on treatment, the feces were very thin and watery, but by the third and fifth days, respectively, they were formed and continued to be so throughout the remaining eleven days of the experiment. The controls likewise recovered, except that the recovery time was delayed.

Group 4 contained 3 pigs. To 1, the drug was administered at the rate of 0.68 Gm./10 lb. and to another, 0.94 Gm./10 lb. No drug was given to the third pig of the group. On the third day of treatment, the stools of the treated pigs were soft and semiformed; three days later the stools were normal, and the pigs were released on the ninth day. The control pig failed to improve and was subsequently placed on treatment with others comprising group 5.

Group 5 contained 5 pigs. Four were treated and 1 left as the control. The control died of infectious enteritis on the tenth day of the experiment and the treated ones were released on the twelfth day.

Group 6 contained 6 pigs, 5 treated and 1 control. The control died of infectious enteritis on the sixth day. The drug was administered by capsule at the rate of 0.62 Gm./10 lb. to 0.70 Gm/10 lb. One treated pig died on the fourth day without showing any signs of improvement. A definite enteric infection, characterized by diphtheritic and necrotic inflammatory reaction, was observed on autopsy. The remainder of the group were released by the tenth day, since their stools appeared quite normal for two and three days previous to their release.

Group 7 contained 7 pigs, averaging 72.7 lb. each. Five were treated, each receiving an average of 0.90 Gm./10 lb. of the drug per day. The drug was mixed with a small amount of swill at the morning and evening

TABLE 2.—Sulfaguanidine in Infectious Enteritis of Swine
Summary of Experiments in Part I.

Group No.	Range in Wt. lb.	No. Swine Treated	No. Control Swine	Daily dose* Gm./10 lb.	Results No. Recovered	Results No. Deaths	Results No. Not Improved
1	22	1		0.90	1		
	20		1				1
2	8—16	7		0.82—1.62	7		
	9—15		7		2	5	
3	13—33	2		1.1—1.84	2		
	22—40		2	0	2		
4	21—56	2		0.68—0.94	2		
	25		1	0			1
5	21—46	4		0.82—1.24	4		
	36		1	0		1	
6	20—48	5		0.6—0.7	4	1	
	47		1	0		1	
7	65—80	5		0.09	5		
	60—74		2	0	1	1	
8	55—70	7		1.0	4	3	
	45—75		3	0		3	
9	15—29	7		0.52—0.86	1	6	
	16—19		4	0		4	
10	11—24	5		1.0		5	
	12—20		4	0		3	1
11	21—43	5		1.0	4	1	
	20—39		4	0		3	1
12	30—45	2		1.0	2		
	30		1	0		1	
Total	Treated	52			36 (69%)	16 (31%)	
	Controls		31		5 (16%)	22 (71%)	4 (13%)

*Dose divided between morning and evening.

feeding. One of the group was released on the sixth day of treatment, 3 on the eighth, and 1 on the ninth day. One of the controls was released as normal on the eighth day, having shown marked improvement for four days. The other control died of infectious enteritis on the sixth day. The controls were maintained separately from the treated pigs at all times.

Group 8 contained 10 pigs which came from a herd containing approximately 180 shotes, among which there were many cases manifesting signs of an intestinal disease. The 7 pigs which were to receive treatment ranged from 55 to 70 lb. in weight. They were given sulfaguanidine at the rate of 1.0 Gm./10 lb., mixed with their food. One died on the second day of treatment, another on the fifth day, and a third on the sixth. All revealed lesions characteristic of infectious enteritis. Four were released, 1 on the eighth, 2 on the ninth, and 1 on the tenth day. The controls all died of infectious enteritis before the ninth day.

Group 9 contained pigs suffering from a complication of an infectious enteric disease and pneumonia following an attack of influenza. Seven received sulfaguanidine and 4 served as controls. Only 1 treated pig survived. It received 0.86 Gm./10 lb. of the drug per day by capsule and was released on the ninth day. The doses given the others were somewhat smaller, ranging from 0.52 Gm./10 lb. to 0.68 Gm./10 lb. None of the latter ever showed any indications of improvement, and all the pigs, including the controls, died by the twelfth day.

Group 10 is of especial interest because it represents a series of cases of infectious enteritis in the late stages of the disease. These pigs were in poor physical condition, characterized by extreme emaciation, dehydration, weakness and a watery diarrhea containing tissue shreds of diphtheritic and necrotic exudative nature. Their appetites were only fair, and they lay huddled together in a corner of the pen most of the time. Five received sulfaguanidine by capsule at the rate of 1.0 Gm./10 lb. and 4 were left untreated. By the fourth day of treatment, 2 of the treated pigs and 2 of the controls were dead, and by the ninth day, 2 more of the treated and another of the controls had died. The stool of the fifth treated pig (13) was formed on the tenth day and continued to be formed at each voiding until the pig succumbed, twenty-three days after the beginning of the experiment. At autopsy, a low-grade enteritis and a terminal pneumonia were observed. One control was still alive when pig 13 died. Its condition had not changed much from the time of admission.

Group 11 is also of especial interest since the cases comprising it represent an acute stage of the disease. The general physical condition of these pigs could well be described as fair to good. They were active and possessed fair appetites. The particular clinical sign manifested was a marked diarrhea containing much blood. They were typical of cases described as "bloody scours." Five were treated and 4 left as controls. The drug was administered at the rate of 1.0 Gm./10 lb. by capsule for the first six days and mixed with the feed for the next four days. The stools of the 4 treated pigs that survived were formed on the sixth day and on each day thereafter. These pigs were released on the tenth day. One of the treated pigs died on the second day of the experiment. All of the controls died, 2 on the second, 1 on the ninth, and 1 the twenty-fifth day. The postmortem lesions in these cases were described as a hemorrhagic type of inflammation of the large bowel.

Group 12 contained 3 pigs. The 2 that were treated received sulfaguanidine at the rate of 1.0 Gm./10 lb. mixed in a small amount of milk. Both made marked improvement and were released on the eighth day. The control died on the sixth day.

The results of the study on the value of sulfaguanidine in the treatment of infectious enteritis where controls were included are summarized in table 2. Of the 52 pigs that received treatment, 36 (69%) were released as recovered and 5 out of 31 (16%) of the controls recovered. Eleven pigs that were sick with swine influenza and pneumonia as a complication of the enteric disease, and 9 that were sick with enteritis in a very advanced and chronic form are included in these statistics. They represent types of cases that could not reasonably be expected to respond to this therapy.

Part 2

This part of the report deals with the treatment of infectious enteritis in swine under field conditions. It was necessary in most instances to forego setting aside some of the affected pigs to serve as untreated controls because it was not convenient in some cases and was impossible in others. However, the presence of the disease was assured before therapy was instituted. Postmortem examinations were conducted on two or more pigs prior to treatment and, in most cases, on those that died during the course of the treatment. Under this arrangement, a total of 446 swine was treated with sulfaguanidine. Daily observations were made on 133 of this number, whereas the remainder were so situated that only periodic inspections by the authors or the local veterinarians were feasible. For this reason, the 2 groups will be discussed separately as sub-groups *A* and *B*, respectively.

Sub-group A.—Table 3 summarizes the data on sub-group *A*. The animals in this sub-group varied considerably in age and weight and in clinical manifestations of the disease. Some of the pigs were sick with the acute form of infectious enteritis, some with the chronic form and others were in between. In general, the feces returned to a normal character by the fourth or fifth day of treatment, and the pigs were usually released as recovered by the seventh day or

the eighth. Generally, the pigs were not released until after the stool had appeared normal for three or four days.

Although the animals treated with the drug in the dosage range of 0.5 Gm./10 lb. responded to treatment quite satisfactorily, those receiving it in the higher ranges seemed to improve a little more rapidly if one were to judge from the character of the feces and physical condition of the pigs.

The method of drug administration varied from once a day to twice a day, either by capsule or in small amounts of mash or thick swill. In this limited number of cases, no definite evidence was obtained which indicated an advantage of one method over another. If pigs will eat sufficient feed to receive the drug, the effectiveness is in no way impaired. On the other hand, in those animals that either refused to eat or ate only sparingly, it was found advisable to administer the drug by capsule, treating each pig separately. This was continued until they were "back on feed," and then the drug was mixed with the feed.

TABLE 3.—Sulfaguanidine in Infectious Enteritis of Swine
Summary of Part 2 A.

Range in Daily Dosage Gm./10	Range in Wt. Lb.	No. Swine Treated	Results		
			No. Recovered	No. Died	No. Not Improved
0.30—0.50	40—101	18	18	..	..
0.51—0.75	42—200	50	43	7	..
0.76—1.0	30—200	37	35	2	..
1.01—1.5	24—200	23	21	1	1
1.51—2.0	47—200	5	5	..	..
Total	.....	133	122	10	1
			(92%)	(7.5%)	(0.75%)

Sub-group B.—It seemed desirable to evaluate the effectiveness of the drug under conditions such as might obtain in a general practice. The results of this phase of the study are reported in a series of protocols and constitute sub-group *B* of part 2 of this report. These studies were carried out with the coöperation and assistance of several practitioners. A total of 333 swine on six different farms is included.

Protocol 1 (Art. B.).—On or about July 1, 1941, 115 recently weaned pigs were pastured together in a plot containing a luxuriant stand of alfalfa and were given a supplemental feed consisting of ground corn and oats and a small amount (15-20 gal.) of skimmilk. They had been growing and developing satisfactorily until September 2, when one of the pigs was found dead. Postmortem examination revealed a diphtheritic enteritis. Inasmuch as another similar plot was available, and on the assumption that the disease might be curtailed from spreading among the group, these pigs were transferred to the other plot immediately. Three days later, 2 of the pigs were seen to pass diarrheal stools. They were removed and isolated in a pen several rods distant. On the following day, a new case developed. It was immediately segregated.

At this time, September 6, 2 of the sick pigs were started on a course of treatment with sulfaguanidine and 1 was left as a control. The dose of the drug was 1.0 Gm./10 lb./day, mixed with a small amount of milk, morning and evening. At the beginning of treatment, all 3 pigs had a profuse diarrhea containing considerable amounts of blood. They were weak and considerably dehydrated. The control died five days later, and at necropsy a hemorrhagic and diphtheritic inflammation of the large intestine was observed. The treated pigs were released as recovered after seven days of treatment.

On September 13, 4 new cases had developed in the large herd. They were removed and started on a course of treatment with sulfaguanidine. The dose was 0.7 Gm./10 lb., and the mode of administration was in milk. They all manifested a bloody diarrhea when treatment was begun. Three days later, the feces began to form and in another three days they were normal. The treatment was continued another two days.

On October 5, 20 pigs, all affected with enteritis, were sorted out from the herd, moved to a smaller pen, and started on a course of treatment with sulfaguanidine.

The drug was given at the rate of 0.6 Gm./10 lb., mixed with the feed. At the same time, 13 other similarly affected pigs were segregated to serve as controls. One pig in the treated lot and 3 in the control lot died within the next 4 days. On October 14, 14 of the treated pigs had recovered sufficiently to be released. Since the 10 controls were gradually becoming weaker, they were turned in with the 5 that had been receiving the drug since October 5. This lot of 15 was then placed on treatment, the drug being administered in the feed at the rate of 0.5 Gm./10 lb. All but one of the lot were released as cured on October 24. The pig that died was one that had served as a control.

The condition in the general herd, in so far as the disease was concerned, was not improving. From time to time, pigs were becoming sick with infectious enteritis, and it was practically impossible to segregate them in small lots or units where treatment could be given. For this reason, it was decided to place the entire herd on treatment with sulfaguanidine. The herd at this time numbered 108 animals, at an average estimated weight of 125 lb.

The drug was administered at the rate of 0.04 Gm./10 lb. once a day, mixed with milk. This was a small dose, but it was all that was available at the time. In four days, the supply of the drug was exhausted and not until four days later was it possible to continue treatment. The dose was now increased to 0.16 Gm./10 lb. and continued for six days. Six deaths occurred during this time. At the end of the six-day period, 10 pigs which were voiding bloody stools were removed and treated with a still larger dose, 1.2 Gm./10 lb. Treatment on this lot was continued for ten days, whereupon 9 were released and then the tenth succumbed. All treatment was discontinued on or about November 24. Between November 24 and January 10, 3 cases of enteric disease occurred among the pigs of this herd; all recovered spontaneously. The condition of

TABLE 4.—Blood, Fecal, and Urine Sulfaguanidine Values
(Average and Range)

Range of Daily Dosage Gm./10 lb.	Blood				Feces				Urine			
	No. Animals	No. Samples	Free mg. per 100 cc.	Total mg. per 100 cc.	No. Animals	No. Samples	Free mg. per 100 Gm.	Total mg. per 100 Gm.	No. Animals	No. Samples	Free mg. per 100 cc.	Total mg. per 100 cc.
0.43—0.54	4	11	0.89 0.56—1.65	1.45 0.81—3.00	4	12	160 57— 406	189 62— 497				
0.60—0.75	8	19	1.70 0.70—4.24	3.45 1.28—9.0	9	18	357 81— 594	389 134— 666	1	1	122	159
0.80—1.10	8	20	2.01 0.86—6.9	3.65 1.38—13.6	23	32	405 104— 1057	435 104— 1220	9	11	160 106— 305	290 137— 588
1.2 —1.5	2	2	6.9 6.6 —7.2	9.8 9.6 —10.1	4	8	457 217— 928	510 331— 956	4	6	656 144—1350	1035 302—2060
2.0	1	9	3.05 1.5 — 6.0	4.97 3.1 — 6.8	1	9	574 373— 1024	636 430— 1139	1	3	312 130— 475	455 180— 695
3.0	1	2	6.2 6.0 — 6.4	12.3 9.3 —15.3	1	2	1175 1090— 1175	1290 1150— 1430	1			
4.0	1	4	8.6 4.5 —12.8	12.8 8.5 —19.6	1	4	1316 714— 2930	1383 761— 2970	1	3	486 400— 620	783 655—1000
5.0	2	10	4.8 1.6 — 7.9	7.6 4.6 —12.4	2	9	9115 1360—20880	9215 1420—20900	2	8	1002 98—2650	1542 422—4600

the pigs on January 10 was satisfactory, and except for 2 whose growth was considerably stunted, the pigs averaged approximately 240 lb. each.

Protocol 2 (H. H. B.).—On Sept. 6, 1941, this herd contained 167 pigs ranging in weight from 30 to 100 lb. About 20 of them were clinically sick and displayed symptoms characteristic of infectious enteritis. One was destroyed and showed diphtheritic and necrotic lesions of the large intestines.

The previous history of this herd revealed that on July 5 the herd consisted of 242 pigs. They were all given the serum-virus treatment on this date as a preventive against hog cholera. On or about August 17, 2 pigs in the herd were sick and thereafter many others sickened and many died. The loss from August 17 to September 6 was 75 pigs.

On September 6, 10 sick pigs were placed in a separate pen in the swine barn and started on a course of treatment with sulfaguanidine. One pig received the drug at the rate of 0.6 Gm./10 lb.; 2 at 0.7 Gm./10 lb.; 2 at 0.9 Gm./10 lb., and 5 at 1.0 Gm./10 lb. divided between morning and evening and administered by capsule. Treatment was continued for six days, at which time they were released as normal. During the same period, 4 in the general herd died. A second series was treated on September 14. Fifteen comprised this group and received the drug at the rate of 0.7 Gm./10 lb. mixed in milk. After three days, the dose was reduced to 0.5 Gm./10 lb. and continued for four days. One pig died on the second day of treatment, but all the others were released as cured. Four pigs in the general herd died during this period and new cases were developing. It was necessary to discontinue any further trials in this herd.

Protocol 3 (M. C.).—On Oct. 15, 1941, this herd contained 1 boar, 6 brood sows with 36 suckling pigs, and 38 spring pigs. On October 16, several of the spring pigs were seen to pass blood with the feces, and two days later 20 of this group were definitely sick. Within the next three days, 6 of the group died. Sulfaguanidine was administered to the 14 sick pigs, beginning on October 21. The dose was as low as 0.08 Gm./10. Seven deaths occurred within the next twenty-four hours. The remainder of the group, plus 2 more added on the fifth day of treatment, received the drug for fourteen days, whereupon they were released as cured. At this time, 2 of the brood sows and 8 of their pigs were showing symptoms of enteric disease. The pigs were weaned and placed on sulfaguanidine at 0.13 Gm./10 lb. Treatment was discontinued after three days and the pigs released. The brood sows received a similar course of treatment, except that it was continued for six days before they were released. On November 22, 7 of the spring-farrowed and 6 of the fall-farrowed lot were sick with the disease. They were started on treatment immediately, and after six days were returned to the feed lot as recovered. The dose was 0.1 Gm./10 lb.

Protocol 4 (A. P.).—This herd contained brood sows and 73 suckling pigs 3 to 5 weeks of age. Approximately, 60 per cent of the suckling pigs were sick and, according to the owner, the sickness was spreading rapidly among the small pigs. Postmortem examination of 2 of the pigs revealed lesions suggestive of a complication of cholera and infectious enteritis. The pigs and their dams received a course of treatment with cholera antiserum and virus. Four days later, from the standpoint of the hog cholera, the herd appeared much improved. A postmortem examination of one at this time showed a marked ulcerative gastritis and diphtheritic and necrotic colitis and some lesions suggestive of hog cholera. It seemed advisable to treat the group with sulfaguanidine, and on October 26, 62 of the small pigs were given the drug in milk at the rate of 0.6 Gm./10 lb. The treatment was continued for nine days, with the result that 12 succumbed and 50 were released since they showed marked improvement.

Protocol 5 (E. L.)—The swine population on this farm numbered 70 (25 at 70 lb. and 45 at 30 lb.) At the time of treatment with sulfaguanidine, 10 of the former and 15 of the latter were definitely sick with infectious enteritis. Before any treatment was begun, however, 2 of the sick pigs were de-

stroyed and examined postmortem. The mucous membranes of the cecum and colon of both animals were almost entirely covered by diphtheritic and necrotic exudative material. The larger pigs were separated from the smaller ones and treated separately. They received 0.3 Gm./10 lb. of the drug mixed with the feed, and the smaller pigs received 0.25 Gm./10 lb. Four of the larger and 2 of the smaller pigs died before the treatment was discontinued on the eleventh day.

While it appeared that the pigs recovered from the inflammatory disease of the intestine, yet they failed to grow and develop satisfactorily. Their plane of nutrition and the management they were receiving left much to be desired. About three weeks after they were released from treatment, several deaths occurred among them and within a ten-day period 17 succumbed. The specific cause for these losses was not determined.

On or about October 20, 84 pigs averaging 40 lb. each were purchased at a local sales pavilion and placed in the pasture with the swine native to this farm. Within a week or ten days from this date, some of the newly purchased animals were sick, and by November 10, 20 pigs had died. At this time, medical assistance was secured. In the opinion of the local veterinarian, hog cholera and necrotic enteritis existed as complicating factors responsible for the current condition of a large proportion of the drove, exclusive of the boar and brood sows. The latter had been given a protective treatment against hog cholera a year or more before. As a result of his findings, the pigs were given an intramuscular injection of hog-cholera antiserum, and on the same day they were started on the so-called salt treatment for necrotic enteritis.

TABLE 5.—Sulfaguanidine Toxicity Studies.

Pig No.	Body Weight lb.	Daily Dose Gm./10 lb.	Day of beginning of inappetence	Clinical Observations	Day of Death or Destruction
912	60	2.0	29	Locomotor incoördination and later complete paralysis	31
914	85	3.0	5	Vomits after drug administration beginning on 5th day; marked weakness on 5th day.	9*
913	50	4.0	6	Locomotor incoördination	12*
915	100	5.0	5	Locomotor incoördination, lassitude, weakness	23*
917	87	5.0	4	Locomotor incoördination, weakness	9*
918	84	7.0	4	Locomotor incoördination	5
916	60	10.0	4	Locomotor incoördination, dyspnea, weakness, paralysis	8

*= Moribund and destroyed.

Protocol 6 (E. B.).—The history of this drove prior to the time sulfaguanidine was used on some of the pigs comprising it is as follows: Early in October, 1941, the drove contained 1 boar, 17 brood sows, 8 shotes, and 40 small pigs recently weaned.

On November 14, since the enteric disorder was noticeable throughout the drove, it was decided to separate the drove into 3 different lots. Lot *A* contained 25 of the recently acquired pigs. They received sulfaguanidine at the rate of 1.5 Gm./10 lb., mixed in the feed. The treatment was continued for eleven days, when 24 were released as recovered; 1 had died the third

day after the treatment began. Thirty-nine of the same group of pigs made up lot *B*. These were continued on the "salt treatment." Of this lot, 18 died within the first ten days; the remainder were discharged as cured after eighteen days of treatment. Lot *C* contained 40 pigs, raised on the farm. These were placed on an "alkaline treatment." Eighteen succumbed during the first ten days, and the remainder were released after two weeks on treatment.

On or about December 1, 5 of the brood sows were manifesting symptoms suggesting the presence of the infectious enteric disease which had been so prevalent among the younger animals. These 5 were started on treatment with sulfaguanidine at 0.1 Gm./10 lb.; all died within a week. The remainder of the sows were sold for slaughter. It is believed that the drug dosage in this case was not adequate.

Part 3

This part deals with the concentration of sulfaguanidine in blood, feces, and urine. The method of Bratton and Marshall[12] for the determination of sulfanilamide was used for the sulfaguanidine determinations. In order to obtain equilibrium values, specimens for analysis were not collected until at least twenty-four hours had elapsed after administration of the first dose. Table 4 is a summary of the data obtained. The relationships found between the blood and fecal values are similar to those first reported by Marshall and coworkers[2] for man, as well as those found by Cameron and McOmie[9] for pigs, and by Thorp and Shigley[13] for calves.

There does not seem to be a relationship between the consistency or character of the feces and the drug concentration. This might be explained on the basis of the absorption of appreciable amounts of the drug by the gastrointestinal tract, as indicated by the relatively high concentration of the drug in the urine. Apparently, a fairly high percentage of the drug is eliminated *via* the urine.

Although the percentage of the total drug present as the acetylated or combined form in the blood, feces, and urine varied considerably with different animals and different specimens, the average values found are approximately 40 per cent, 8 per cent, and 35 per cent for blood, feces, and urine, respectively. The percentage of the drug present in the feces as the inactive or acetylated form is relatively low.

In general, the urine was clear when voided, provided the total drug concentration was not above 500 mg. per 100 cc. Within this range of concentration or slightly above it, the crystals present in the urine were chiefly the acetylated or combined form of the drug, which is much less soluble than the free drug. Indications were obtained that the free form of the drug is soluble in urine as voided to the extent of 400 to 600 mg. per 100 cc. and the combined form approximately 150 to 200 mg. per 100 cc. These values are higher than those reported by Marshall and coworkers[1] for the solubilities of these compounds in urine at 37 C. The question of the solubilities of the combined and free forms of the drug in feces and urine, as well as the relative amounts of the drug eliminated *via* the feces and urine, needs further study.

Part 4

Preliminary toxicity studies were carried out on 7 normal pigs varying in weight from 50 to 100 lb. The dosage used varied from 2 to 10 Gm./10 lb./day. The drug was administered in divided doses (morning and evening) in small amounts of feed until the animals refused to eat, after which it was suspended in water and administered (morning and evening) by stomach tube. This was continued until the animals expired or became moribund. In a few instances, it was observed that the pigs vomited after receiving the drug by the stomach tube. Under these circumstances, it was impossible to be certain how much of the drug was retained. However, vomiting occurred only in the later stages of the experiment.

The results of the toxicity studies are summarized in table 5. From the standpoint of the clinical evidence of toxicity, the first manifestation was inappetence, which necessitated administration of the

drug by stomach tube. The inappetence was soon followed by locomotor incoördination characterized chiefly by a stiff upward and forward stride of the posterior limbs, then by a general and progressive weakness which ended in a moribund state. Except for the evidences of kidney involvement, the postmortem findings were in general negative. The kidneys were swollen and somewhat softer than normal. In some cases, white crystals were present in the pelvis of the kidney in appreciable quantities. Microscopic examination of the kidneys revealed tubular degeneration with many tubular casts and in general presented the picture of nephrosis. From time to time during the course of the experiment, blood was collected from each of the pigs for the purpose of making a hemogram. At each examination, the leucocyte count was always considerably above the normal level. Total white cell counts of 30 to 45 thousand were common (12 to 22 thousand, normal). Except for an increase in eosinophiles equal to 3 to 5 times normal, the ratio between lymphocytes and polymorphonuclear leucocytes was not changed. The red cell count and hemoglobin values were not significantly altered.

Discussion and Summary

The results of these studies indicate that sulfaguanidine possesses considerable merit as a therapeutic agent in the treatment of the infectious enteric complex in swine commonly grouped under such terms as infectious enteritis, necrotic enteritis, etc. The therapeutic measures now in general use have not given satisfactory results in many instances. There is great need for an effective agent for the treatment of this disease as it is one of major economic importance.

Sulfaguanidine, when administered morning and evening as a suspension in a small amount of mash or thick swill at the rate of 0.75 to 1.5 Gm./10 lb. body weight per day (0.16 to 0.33 Gm./kg.), gave favorable results. Those animals which would not eat were given the drug by capsule until they would consume sufficient food to permit the administration of the drug in a portion of the feed. In order to obtain high fecal levels of the drug as early as possible, particularly in the acute cases, the first dose given was always twice the usual dose of the drug. As a rule, the physical condition of the animals began to show improvement by the third to fifth day and the character of the feces returned to normal by the fourth to sixth day. Usually, the drug was administered for a period of six to eight days, which generally included a few days after the stools had returned to a normal consistency.

It is believed that animals which recover from infectious enteritis as a result of sulfaguanidine therapy should not be considered as possessing any significant immunity or resistance to reinfection and that such therapy should be supplemented with sanitary measures to prevent reinfections. A few animals treated with sulfaguanidine have shown remissions. At present, it is not known whether these remissions were due to reinfections or to the failure of the drug to eliminate the causative organisms of the original disease. This phase of the problem needs more extensive study.

Acknowledgements

The authors wish to express their sincere appreciation to Drs. J. N. Campbell, A. H. Schmidt, B. A. Zupp and Professor E. F. Ferrin for their generous coöperation and assistance, and to Dr. B. M. Lyon of the Lederle Laboratories, Pearl River, New York, for the supplies of sulfaguanidine and support toward carrying out this study.

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Corrigenda

In the article entitled "Incidence of Gastrointestinal Nematodes of Cattle in the Southeastern United States" by Dale A. Porter, Sc.D., of Auburn, Ala., appearing in the July issue, Vol. III, No. 8, p. 304, line 26, *insufficient* should read *sufficient*.

In the article entitled "Homogenized Sulfanilamide-in-Oil Intramammary Injections in Bovine Mastitis" by J. V. Kakavas, PhD., C. C. Palmer, D.V.M., M.S., James R. Hay, B.A., D.V.M., and Edward S. Biddle, M.S., Newark, Del., appearing in the July issue, Vol. III, No. 8, p. 274, the first sentence in the second column should read as follows: Little reported that the oral administration of as much as 1,361 Gm. of sulfanilamide per cow failed to destroy the streptococci in the infected quarters.

Change in Reference Style

The American Veterinary Medical Association has adopted a new style of identifying references, namely:

Smith, J. W., and Jones, L. R.: Chronic Myeloid Leucemia in a Hog. Am. J. of Path., *43*, (Dec. 1916): 129-135.

The new style has not been followed consistently in this issue because no changes have been made in material already set up.

The Pathology of Equine Influenza

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EQUINE INFLUENZA constitutes a serious problem in the assembling and conditioning of remounts for military service. The disease is prevalent in remount depots and other places where young, susceptible horses and mules are collected in large numbers. The Veterinary Research Laboratory, Army Remount Depot, Front Royal, Va., was established largely to develop methods for the control of this disease. The studies reported in this paper are designed to aid in the understanding of the basic characteristics of equine influenza. The report is based upon 150 postmortem examinations among over 4,000 cases available for clinical observation.

CLINICAL MANIFESTATIONS

Equine influenza, as seen at this remount depot, is an acute, infectious disease of the respiratory system, characterized primarily by high fever, cough, rhinitis, and frequent pulmonary involvement. Its onset is sudden with elevated temperature (103 to 106 F.), depression, weakness, congested nasal mucosae, and occasionally anorexia. A catarrhal rhinitis which later becomes mucopurulent is soon evident. A dry hacking cough is usually observed. Occasionally, the tendon sheaths and joint capsules exhibit a transient swelling. In a strictly uncomplicated case, the disease terminates uneventfully in a week to ten days.

Some animals show a milder course of influenza than that described above. Complications, however, are common in young animals recently arrived at remount depots. Hemolytic streptococci are the most frequent secondary invaders; complications will depend to a great extent upon where the organisms happen to localize. The most common complication is suppuration of the submaxillary and parapharyngeal lymph nodes. This is characteristic of the condition called strangles, which we believe is secondary to equine influenza.

Pneumonia is a frequent complication and the most serious one; it occurs more often than is generally believed. Its diagnosis in horses is difficult, especially if only the apical part of the lung is involved. A severe neutrocytosis in influenza usually indicates pneumonia—a fact of value in diagnosis. Purpura hemorrhagica is another common complication; it is characterized by the sudden appearance of bright-red petechiae in the nasal mucosa and by sharply demarcated, edematous swellings on the legs and abdomen. The observations of Theiler,[1] Ahmed,[2] Cohrs,[3] and others are in agreement with us on this point. A more detailed consideration of the epizoötiology of influenza and its complications will be given elsewhere.[4]

The blood picture[5] depends upon the nature and severity of complications. During the initial fever, the leucocyte count at first is unchanged, or only slightly elevated. It is then followed by a marked leucopenia of a few days duration, often associated with a relative lymphocytosis. Then, an abrupt increase in polymorphonuclear neutrophile leucocytes occurs. The total leucocyte count may exceed 30,000 cells per

Presented before the Section on Research at the seventy-ninth annual meeting of the American Veterinary Medical Association, Chicago, Ill., August 24-27, 1942.

From the Veterinary Research Laboratory, Army Remount Depot.

[1]Theiler, Arnold: Observations on an Epizoötic Contagious Catarrh of the Respiratory Organs of Equines and its Relation to Purpura Hemorrhagica. Seventh and Eighth Reports of the Director of Veterinary Research, Union of South Africa (1918), pp. 361-393.

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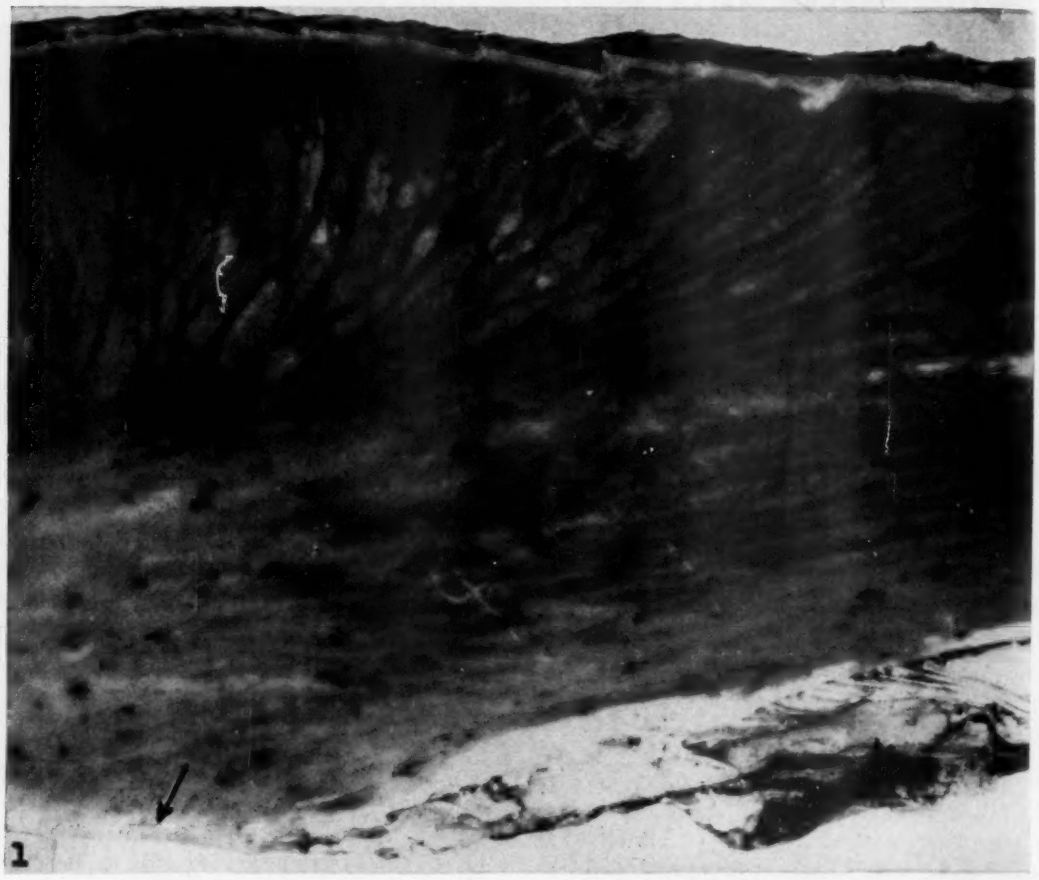


Fig. 1.—Nasal septum, gross specimen. Early equine influenza. Multiple hemorrhages in mucosa. Arrow indicates posterior nares. Army Medical Museum negative 73399.

cmm., over 90 per cent of the cells being neutrophiles. During convalescence, the neutrophile count drops rapidly. A moderate lymphocytosis or monocytosis may occur late in convalescence. Other elements of the blood are not significantly affected in this disease.

ETIOLOGY

Experiments conducted in our laboratory[6] indicate that equine influenza, like influenza in man and swine, is caused by a filterable virus. A mild, uncomplicated, febrile reaction follows exposure of susceptible animals to the virus. On the contrary, it has not been possible to produce the disease with bacterial organisms alone. When the virus is given to susceptible horses together with cultures of streptococci, the typical disease develops. At this station, we have found that hemolytic streptococci are invariably present in the nasal passages of horses sick with equine influenza.

In our series, bacteriological studies were made in 137 cases. Hemolytic streptococci of the Lancefield type C were isolated from the lungs of 128 animals. While frequently not obtained in pure culture, they were the predominating organisms in all cases. No other organisms were isolated with as marked regularity. Streptococci were not recovered from 9 cases. Our findings confirm the belief that hemolytic streptococci are the most important bacterial organisms involved.

[6]To be published.

PATHOLOGY

In our autopsies, 141 were performed on naturally infected cases which terminated fatally. Nine autopsies were on experimentally infected horses, destroyed at intervals during the course of the disease. Among the 141 cases of naturally infected animals, 135 terminated with involvement of the lungs; 6 cases did not show significant pulmonary disease. These 6 cases will be discussed more fully under complications.

Respiratory tract.—In the initial stages, the lesions were confined to the upper respiratory tract and adjacent lymph nodes. Grossly, the nasal and turbinate mucosae were congested, often covered with petechiae and glistening with excess mucus (fig. 1). Microscopically, the epithelium was eroded in some areas, and inflammatory cells, mostly lymphocytes, were seen to invade the submucosa (fig. 2 and 3). As the disease progressed, goblet cells were found to be numerous; later the initial mucous exudate was more and more mucopurulent.

Lymph nodes.—At first, the submaxillary and parapharyngeal lymph nodes were edematous, enlarged, and congested. Later they may develop purulent foci (fig. 4).

Lung.—Ordinarily, the lung was not involved until several days after the onset of the disease. Petechiae in the bronchial mucosa were seen, but they were not constant. The characteristic early lesions in the lung occurred in the apical and ventral parts of the lung (fig. 5). Here a marked edema and thickening of the interlobular septums and distention of subpleural lymphatics were seen (fig. 6). On the pleural surface and cut sections, the involved septums appeared as thick white bands, often as much as 15 mm. in width. A similar reaction was seen around blood vessels and bronchioles (fig. 8). Microscopically, this

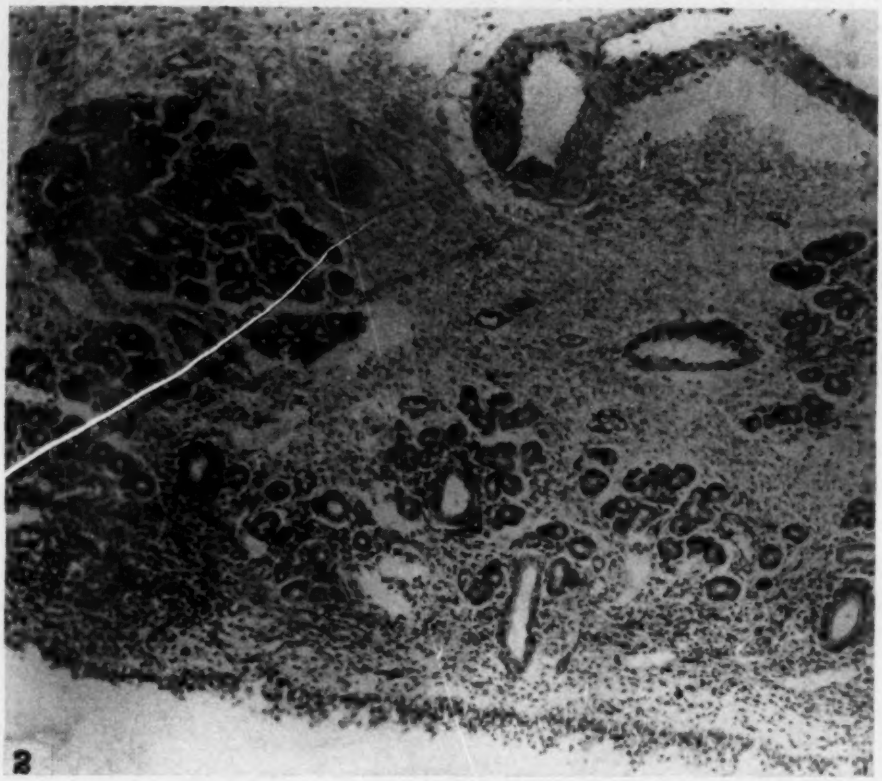


Fig. 2.—Nasal mucosa, x100. Early equine influenza. Desquamation of epithelium, edema and leucocytes in submucosa. Army Medical Museum negative 73387.

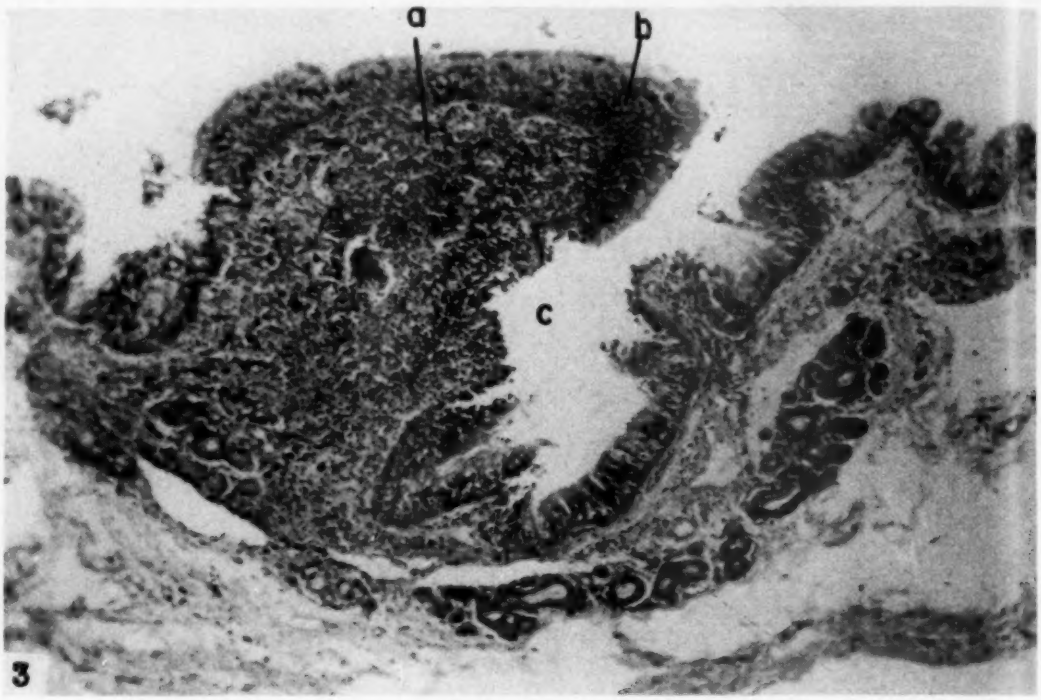


Fig. 3.—Nascal mucosa, x100. Early equine influenza. Leucocytic infiltration of submucosa (a) and mucosa (b); desquamation of epithelium (c). Army Medical Museum negative 73385.
Fig. 4.—Submaxillary lymph node, x600. Early equine influenza. Inflammatory edema in hilux (arrow). Army Medical Museum negative 73414.

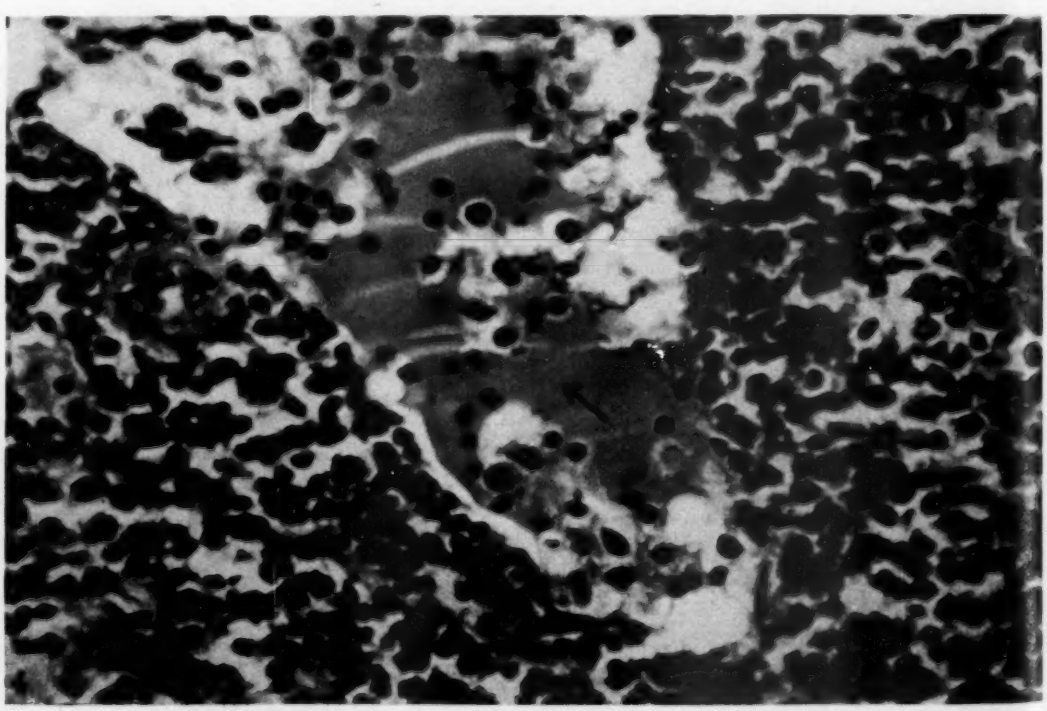


Fig. 5.—Lung. Gross drawing. Early influenzal pneumonia. Note the inflammatory thickening of the subpleural lymphatics in the apex and posterior to the cardiac notch. Army Medical Museum negative 73512.

Fig. 6.—Lung. Photograph. Early influenzal pneumonia. Note inflammatory thickenng of the subpleural lymphatics (arrows). (a) Cardiac notch, (b) ventral border of lungs, (c) Artefact (highlight). Army Medical Museum negative 73402.

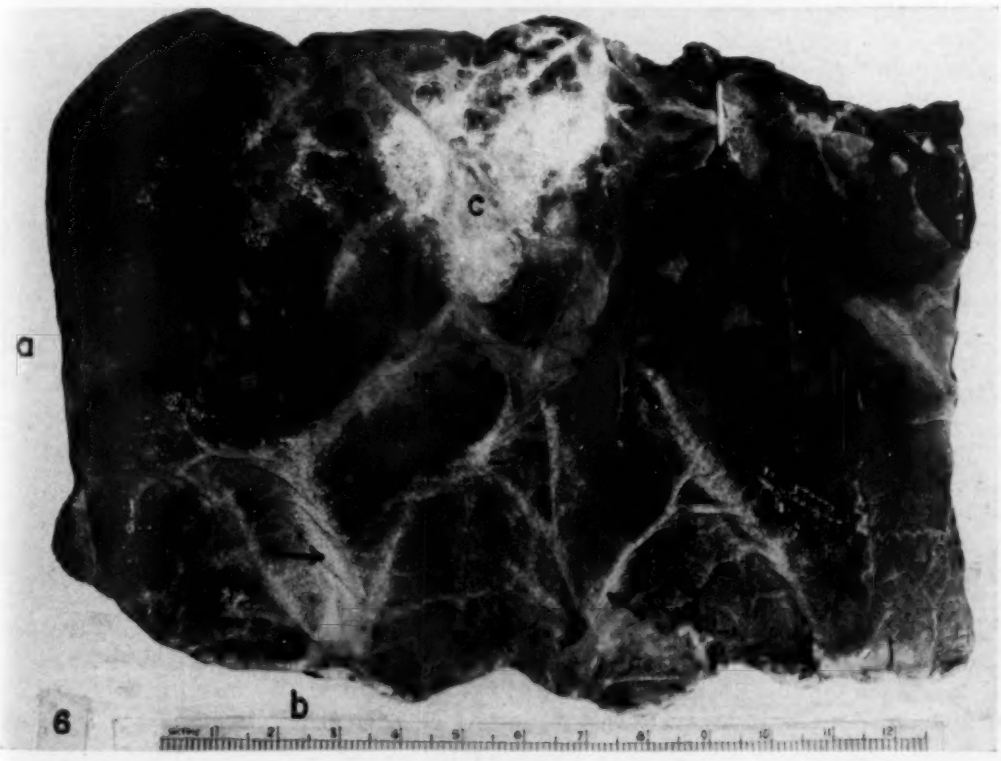


Fig. 7.—Lung. Photograph. Transverse section just posterior to cardiac notch. Equine influenzal pneumonia—convalescence. Note the thickened interlobular septums (arrows). No consolidation was present in this lung. Army Medical Museum negative 73400.

thickening was seen to be due to serous, fibrinous, and purulent exudates. Later, the exudate was organized and remained as bands of white fibrous material extending from the pleura into the substance of the lung (fig. 7). This interstitial inflammation is an outstanding characteristic of equine influenzal pneumonia.

In some cases, however, the interstitial changes were obscured by other lesions, particularly by consolidation of the lung tissue proper. The location of the consolidated areas was quite constant. The apex was affected first, then the ventral portion just posterior to the cardiac notch was involved. The consolidation process tended to spread, but the dorsal parts were last to become affected. The dorsal area was characteristically demarcated from the ventral, consolidated portions. In all cases, both lungs were involved, although rarely to an equal degree.

The type of exudate which predominated in a pneumonic lung varied with the amount of initial infection, the susceptibility of the animal, the complications, and the duration of the disease. The earliest exudate was hemorrhagic. In lungs containing such exudate, the consolidated parts were dark red, heavy, airless, and exuded considerable bloody fluid when cut. It was rare, however, to find pneumonic lungs which were entirely hemorrhagic, but at the height of an outbreak in the winter of 1940 and 1941, a considerable number of cases were of this type. At this time, the disease had a fulminating course. Frequently, an entire lung was found to be hemorrhagic.

As leucocytes became more numerous and red cells broken down, the cut surface assumed a gray, less moist appearance, but remained quite firm and airless. Frequently, different lobules were in various stages of consolidation and resolution, giving the cut surface a "patchy" appearance, with areas of red interspersed among areas of various shades between red and gray.

In later stages of the disease, the complicating action of various secondary invaders was often evident. Grossly, plugs of inspissated purulent material could usually be expressed from cut surfaces of the lungs. Large areas of liquifaction necrosis and confluent abscesses were sometimes present. These were usually in the center of the lung. When necrosis developed, it was commonly accompanied by a foul odor.

Occasionally, circumscribed abscesses

were seen throughout the lung (fig. 17). They were most often subpleural. These abscesses often followed prolonged cases of strangles. We believe that they were metastatic and comparable to the metastatic abscesses seen in the subcutis, kidneys, and other organs in such cases.

Often the pleura was involved. When pleuritis occurred, it was primarily fibrinopurulent in nature. Commonly, the surface was covered with a yellow, shaggy, fibrinous exudate interspersed with much purulent material. The pleural fluid was increased in amount and sanguinous or sanguinopurulent in character and sometimes persisted for considerable periods. In late cases, adhesions between the pleural surfaces were common and sometimes involved the entire costal surface of one or both lungs. It was found that a chronic pleuritis following equine influenza may result in marked compression of the lung due to the accumulation of fluid in the thorax.

Microscopic Picture of the Lungs.—We now consider in greater detail the microscopic picture in the lung. Many stages of inflammation are usually represented in sections from the same lung; this great variability is one of the dominant characteristics of the disease. We have already described the earliest changes which appeared in the interstitial tissues. Hemorrhagic exudate into the interalveolar stroma seemed to be the next step (fig. 10). Then, red cells, serous and fibrinous exudates flooded the interalveolar tissues and alveoli in turn (fig. 11), and leucocytes were soon added (fig. 12). Macrophages also appeared and were filled with blood pigment and debris as the red cells disintegrated. Microscopic fields in which neutrophiles filled all the alveoli were seen, while in adjacent fields mononuclear cells predominated (fig. 15). The alveolar walls usually remained intact, unless abscess formation or necrosis had taken place. At later stages, fibrin and cells were broken down and the exudate tended to become resorbed. When resolution was delayed,

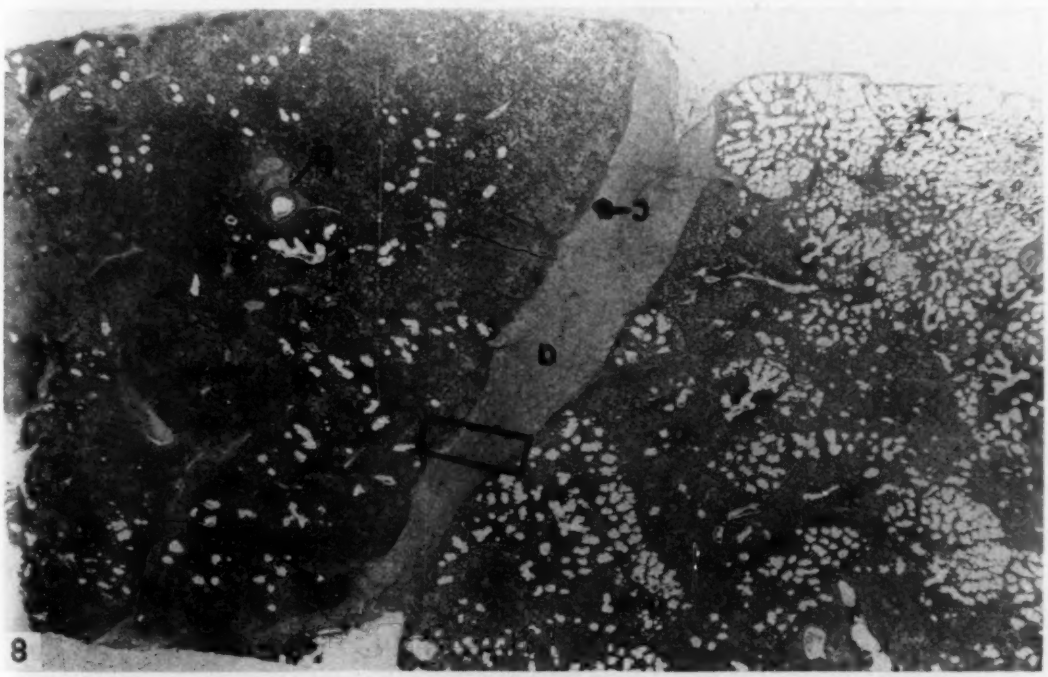
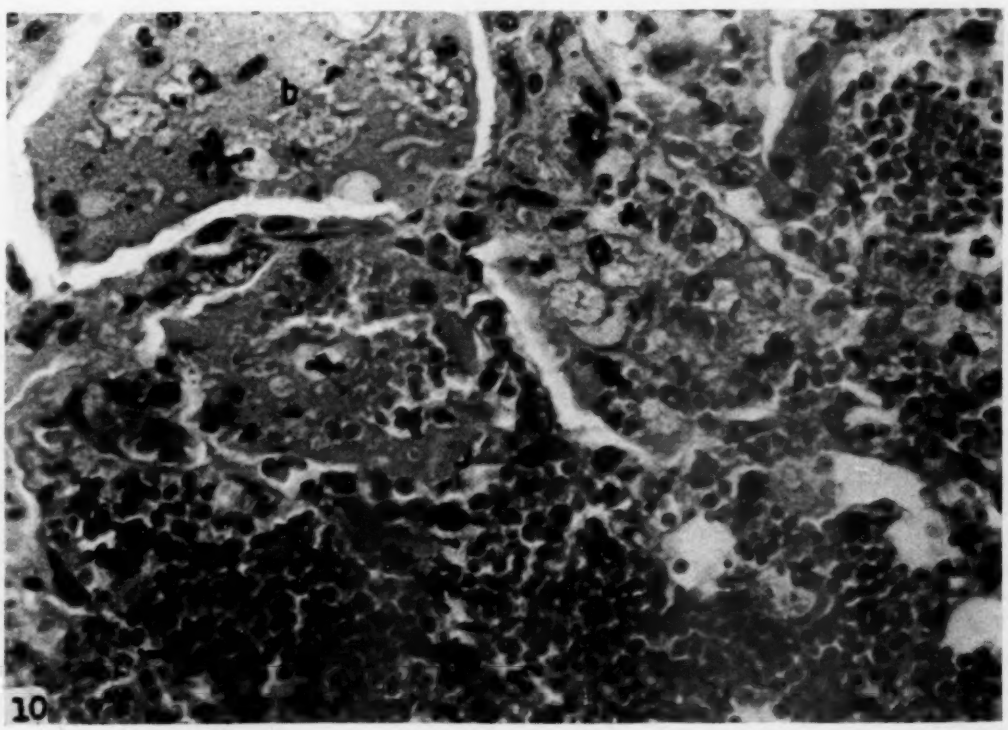


Fig. 8.—Lung. Photomicrograph, x5½. Fatal case of equine influenzal pneumonia. Note the inflammatory edema of the interlobular septum (a) and peribronchial tissue (b). Most densely consolidated area on the right. (c) artefact. Army Medical Museum negative 73419.

Fig. 9.—Lung, x130. Higher magnification of indicated area in fig. 8. Edema, fibrin, and neutrophiles in interlobular septum. Army Medical Museum negative 73383.

Fig. 10.—Lung, x600. Equine influenzal pneumonia; early consolidation. Red cells (a) serum and fibrin (b) in alveoli. Army Medical Museum negative 73413.



there was organization of the exudate. The bronchioles usually contained exudate similar to that in the adjacent alveoli. In some cases, the consolidations were directly peribronchiolar, and appeared to have spread from the bronchi to the alveolar tissue; elsewhere, bronchial involvement seemed to be secondary to the alveolar consolidation. Thrombosis of vessels in the involved areas was commonly observed (fig. 16).

The pleura was frequently thickened by serous and fibrinous exudates with varying degrees of organization and infiltration by neutrophiles and mononuclear cells (fig. 18). The mesothelial layer in some cases was covered by fibrinous and purulent exudate containing bacteria. The connective tissue septums extending into the lung parenchyma from the pleura were similarly edematous and infiltrated with leucocytes.

The lesions in the mediastinal lymph nodes were secondary to those in the lungs. In the earliest stages, these nodes were greatly swollen, edematous, and congested. As the disease progressed, there was an increase in the numbers of immature lymphoid cells, and the germinal centers were no longer discernible. A marked fibrosis of these nodes was seen in longstanding cases. Abscess formation was not common.

Lesions in other organs.—Equine influenza primarily affects the respiratory system but, as has been stated, complications are very frequent in other organs. The extent of nonrespiratory lesions is dependent upon the duration of the disease and the secondary bacterial invaders; they are especially common when septicemia occurs. These lesions are of interest in order to understand the entire picture and in some cases are important from the standpoint of differential diagnosis.

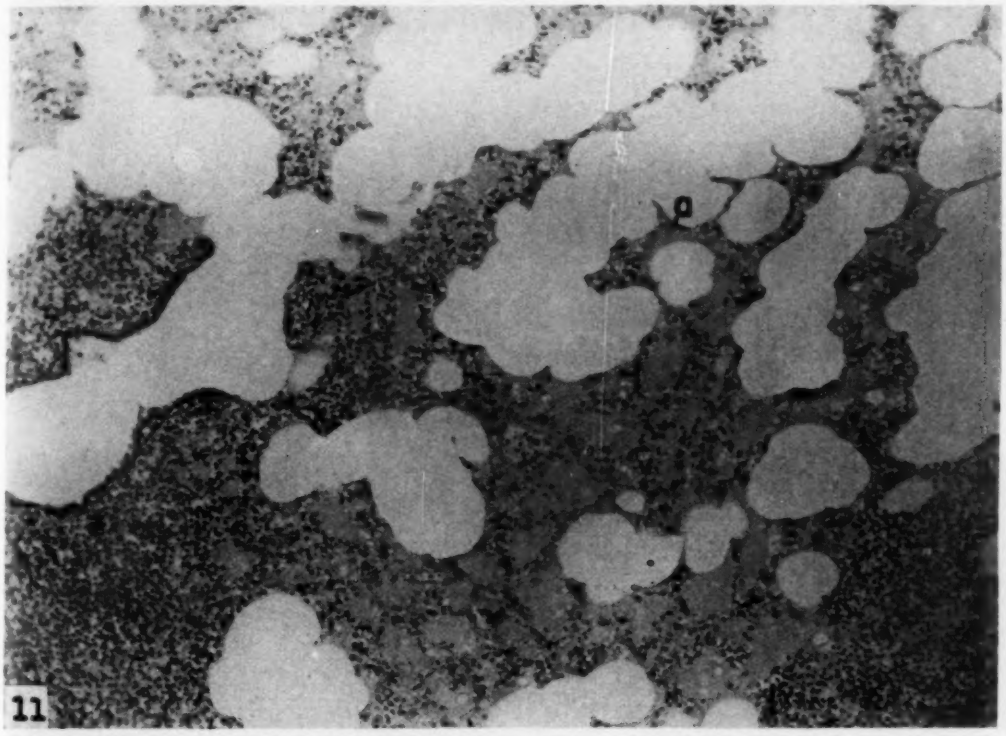


Fig. 11.—Lung, x100. Equine influenzal pneumonia. Interalveolar edema (a) and leucocytic infiltration (b). Early consolidation. Army Medical Museum negative 73391.

Heart.—When pleuritis occurred, the outer layers of the pericardium (including pericardial pleura) were constantly involved. They were thickened, edematous, and covered with an exudate similar to

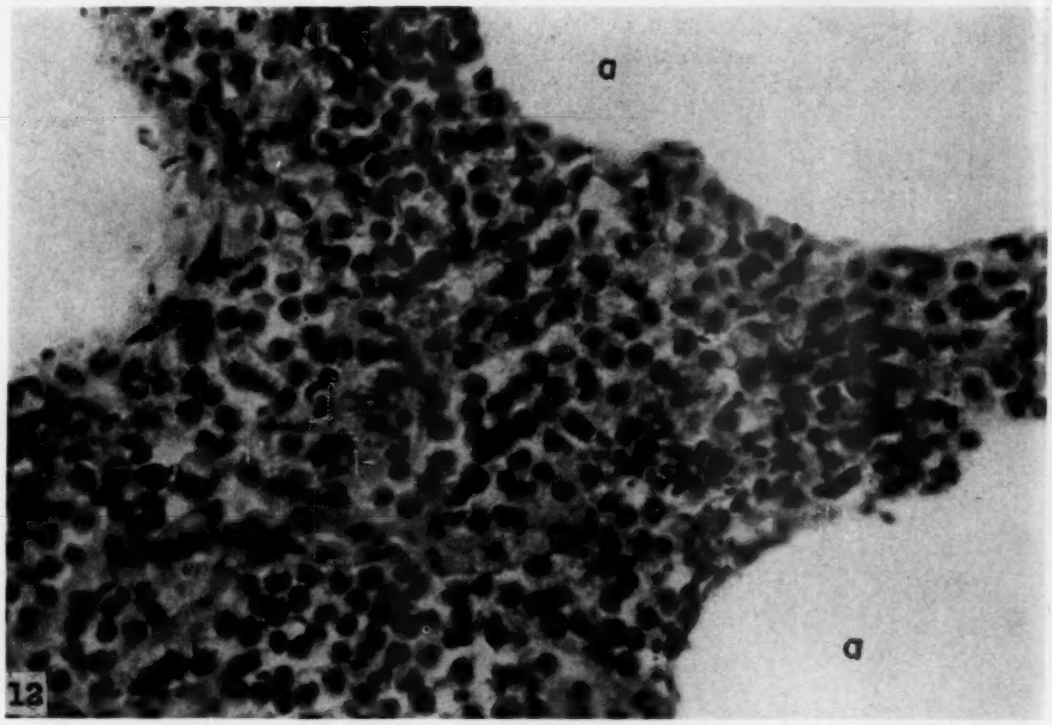
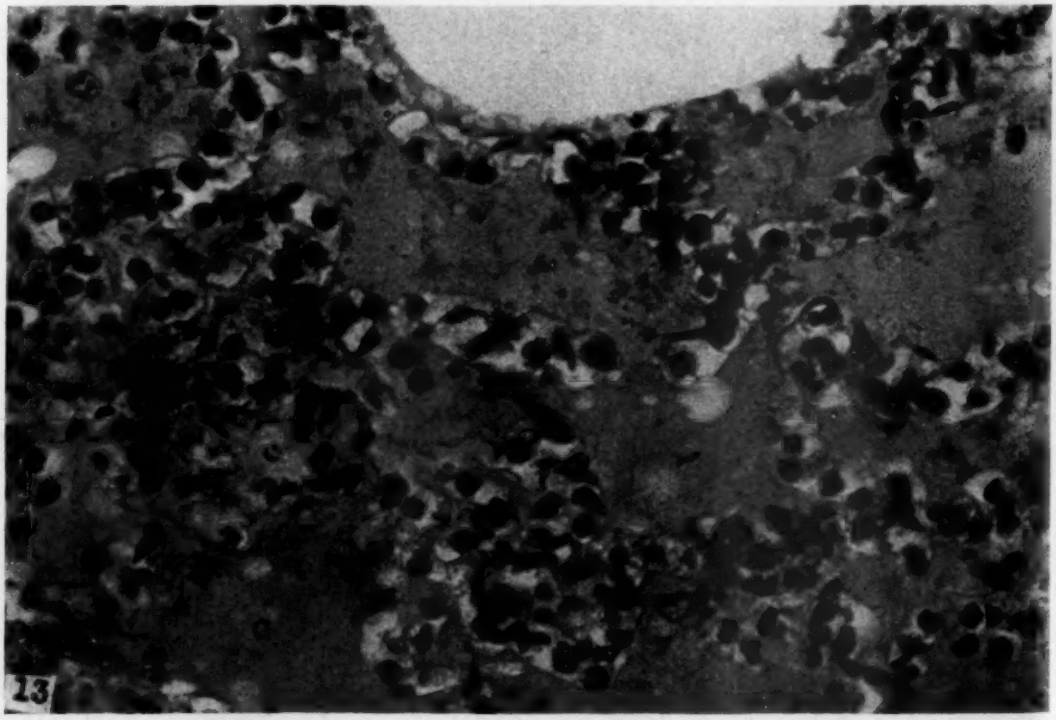


Fig. 12.—Lung, x600. Equine influenzal pneumonia. Interalveolar leucocytic infiltration. (a) alveolus. Army Medical Museum negative 73412.

Fig. 13.—Lung, x600. Equine influenzal pneumonia. Alveoli (a) are filled with serous exudate. Many coccoid bacteria also present. Army Medical Museum negative 73415.



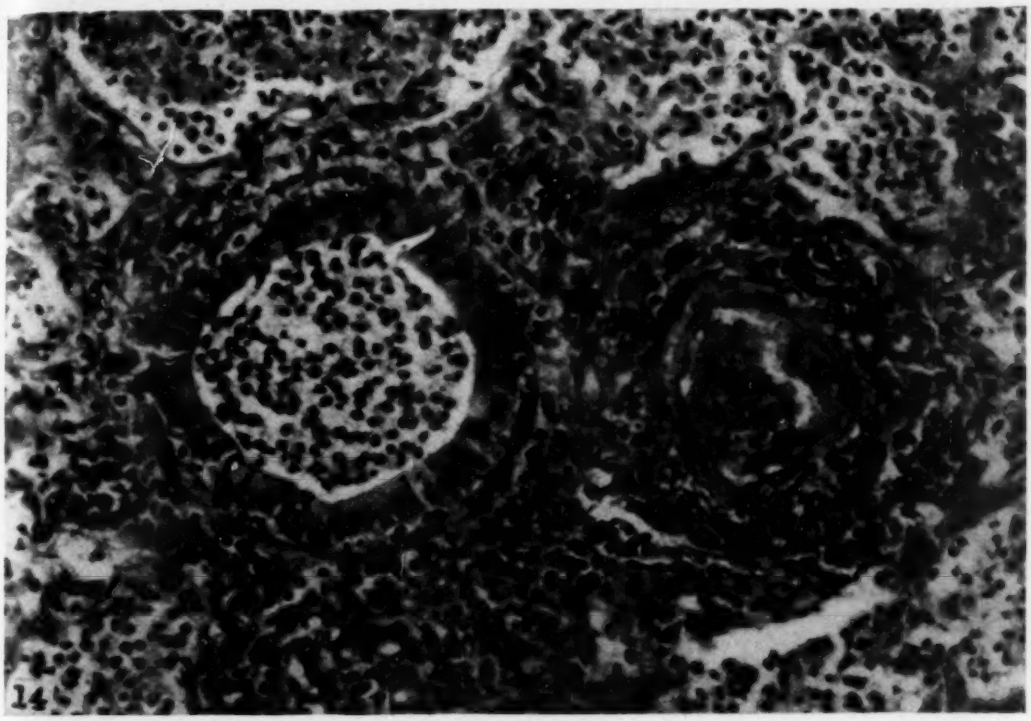
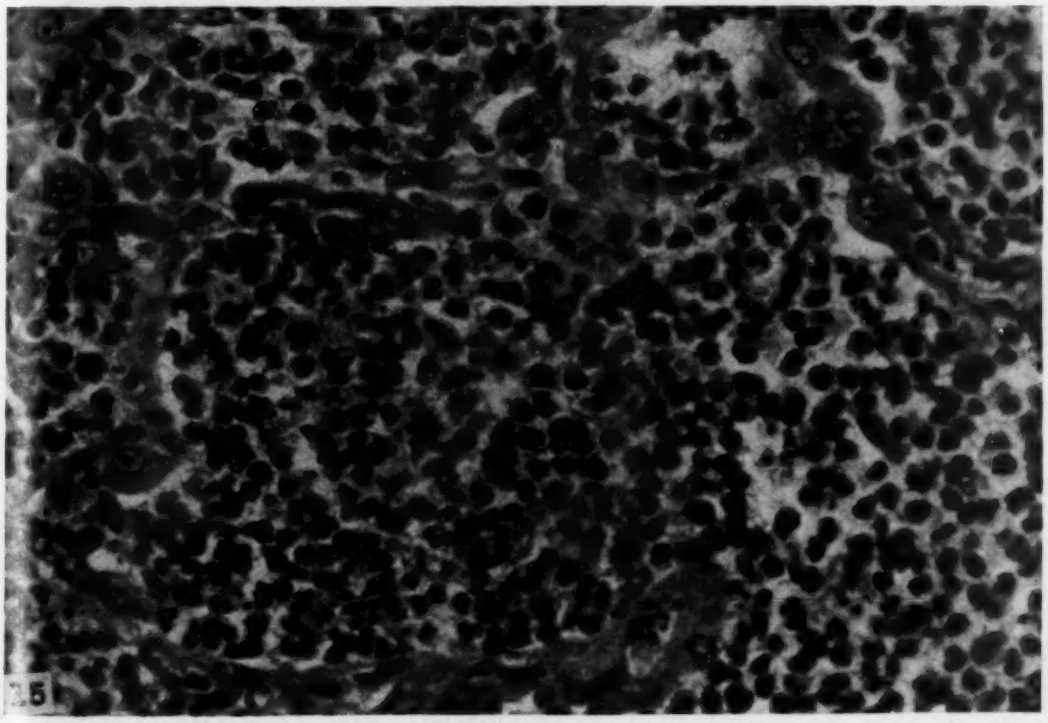


Fig. 14.—Lung, x280. Equine influenzal pneumonia. Neutrophile and mononuclear leucocytes in alveoli and bronchus; also inflammatory thickening of perivascular and peribronchiolar tissues. Army Medical Museum negative 73382.

Fig. 15.—Lung, x600. Equine influenzal pneumonia. Higher magnification of exudate seen in fig. 14. Army Medical Museum negative 73417.



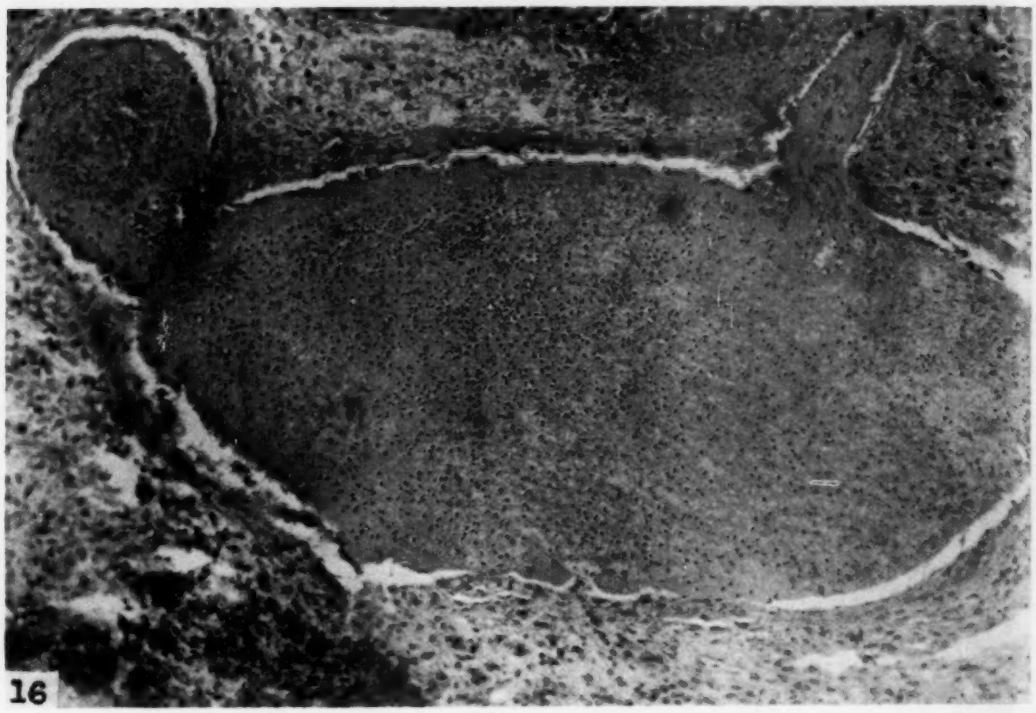
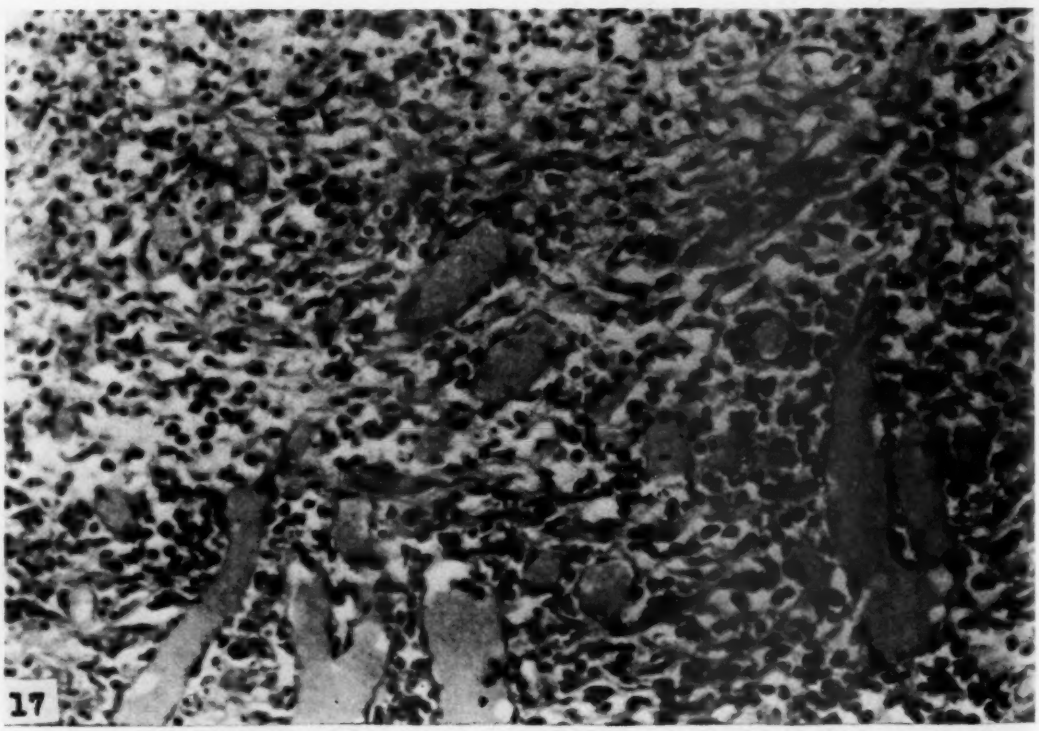


Fig. 16.—Lung, x125. Equine influenzal pneumonia. Thrombus in a consolidated area. Army Medical Museum negative 73418.

Fig. 17.—Lung, x280. Wall of metastatic abscess. Vascular granulation tissue infiltrated by leucocytes, principally mononuclear. Army Medical Museum negative 73416.



that on the pleura. More rarely, the inner layer, *i.e.*, the epicardium and pericardium, were involved with like exudate. Usually, the pericardial fluid was slightly increased in amount and tinged with blood. The epicardium was usually petechiated, especially in the coronary groove. The myocardium was often pale, greyish, and flaccid. Occasionally, the endocardium was involved by dark subendocardial hemorrhages. Large agonal clots were commonly found in the right heart, less often in the left. In one case, we found a large abscess in the septum between the ventricles.

The microscopic picture corresponded to that seen grossly. The mottled areas in the myocardium were due to degeneration and necrosis of some muscle bundles. Subepicardial, subendocardial, and myocardial hemorrhages were seen, occasionally accompanied by small purulent foci. The cardiac lesions result from the heavy burden placed on the heart by the consolidated lungs, the toxemia, and by direct bacterial invasion.

Kidneys.—In acute cases, the kidneys were congested, but otherwise not markedly affected. Microscopically, there was mild,

Fig. 18.—Pleura, x100. Equine influenzal pleuritis. New granulation tissue on each side of mesothelium; fibrinous exudate (a) on surface. Army Medical Museum negative 73380.

Fig. 19.—Voluntary muscle, x30. Purpura hemorrhagica. Extensive inflammatory edema (a) in subcutis; necrosis and inflammatory edema of musculature (b). Army Medical Museum negative 73394.

cloudy swelling. Occasionally, small foci of neutrophiles or round cells were seen in the stroma. In longstanding cases, the kidneys were often enlarged and pale, due to degenerative changes in the lobules. Occasionally, small abscesses were seen grossly. The microscopic picture here is

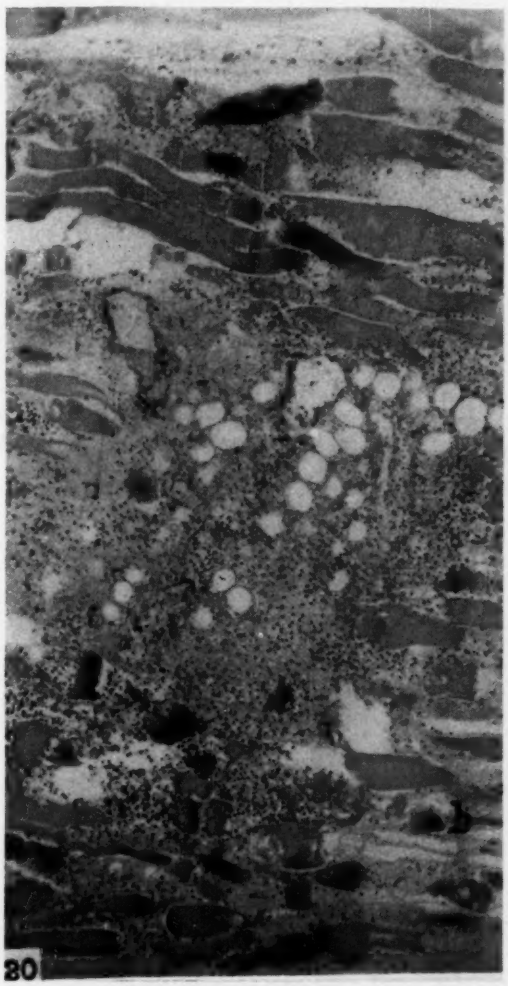


Fig. 20.—Voluntary muscle, x100. Purpura hemorrhagica. Interstitial inflammatory edema (a) and necrosis of muscle bundles (b). Army Medical Museum negative 73388.

one of an interstitial infiltration of leucocytes and hemorrhage with some tubular degeneration (fig. 23). These lesions appear to be metastatic.

Brain and spinal cord.—The brain and cord were not primarily involved, but they may be secondarily affected. Infection spreads directly from abscesses of the upper respiratory tract, parotid lymph nodes, or the gutteral pouch; or bacteria may be carried by the blood stream. The picture was most often one of purulent meningoencephalitis; the spinal meninges were less often involved. Occasionally, large ab-

Fig. 21.—Small intestine, x30. Purpura hemorrhagica. Inflammatory edema of wall, especially marked in muscularis. Army Medical Museum negative 73377.

scesses may develop in the cerebrum (fig. 24). The microscopic lesions are quite characteristic. An intense purulent perivascular reaction was seen in the neighborhood of the abscesses (fig. 25). These reactions serve to differentiate influenzal meningoencephalitis from encephalitis

caused by viruses not associated with bacteria.

Liver.—Grossly, the liver was usually congested, often friable and mottled with areas of pale yellow; lobulation quite distinct. The microscopic picture was that of chronic passive congestion; occasionally, there was central necrosis. Areas of fatty changes were commonly encountered.

Spleen.—Ordinarily, the spleen is not involved, but in some cases when septicemia occurred there was a marked congestion and swelling. The microscopic picture was one of acute "splenic tumor."

Eyes.—Iridocyclitis occurs in a small percentage of equine influenza cases. In one case of our series, an acute purulent iridocyclitis was observed; it affected both eyes one day before death.

Purpura hemorrhagica. — In approximately 14 per cent of our autopsies, purpura hemorrhagica was a complication. The severe subcutaneous edema in these cases often disappeared just prior to death and, for that reason, was not seen at autopsy. Usually, however, characteristic dark, discolored areas remained in the superficial muscles. Microscopically, these lesions consisted of foci of necrosis in the muscle bundles with inflammatory exudates in the surrounding interstitial tissues (fig. 20). The exudate was primarily serous and fibrinous with accompanying collections of neutrophiles. Thrombosed blood vessels were numerous in these areas (fig. 22). In many cases, the intestinal wall was involved in a manner similar to that seen in the subcutis and musculature. When this occurred, the wall was thickened, dark, edematous, and hemorrhagic (fig. 21). Characteristic hemorrhages in the nasal mucosa may or may not be seen at autopsy. In the majority of the cases of purpura, a chronic, localized inflammation with considerable necrosis was found in one or both lungs.

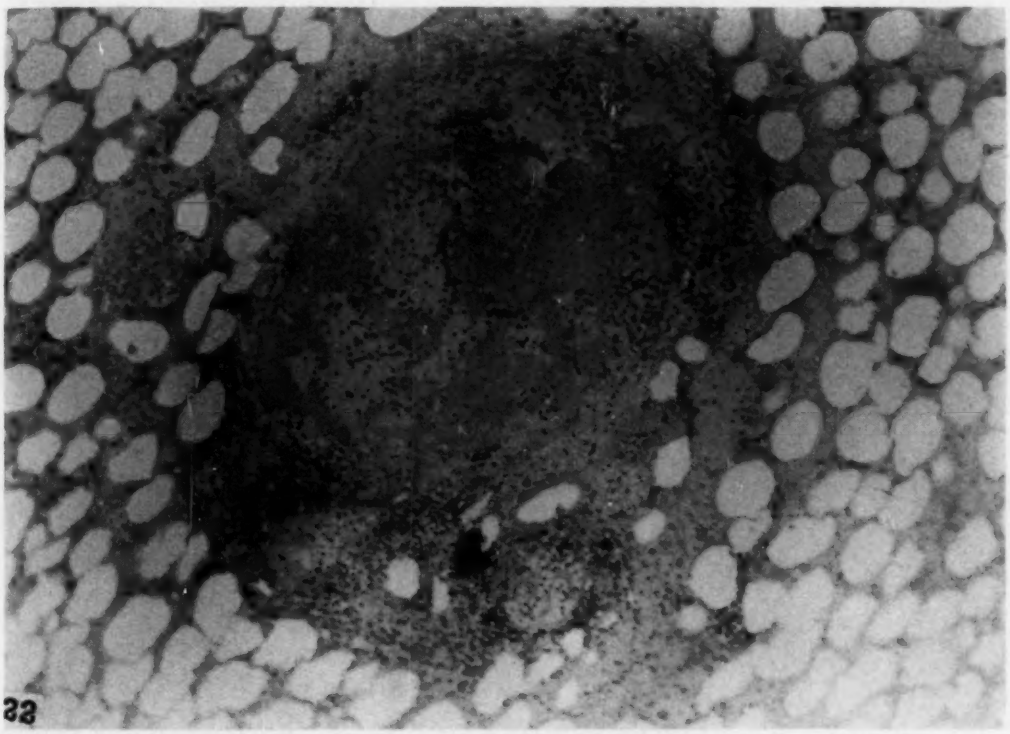


Fig. 22.—Mesentery of small intestine, x100. Purpura hemorrhagica. Thrombus in vein. These are numerous in the mesentery near the intestine. Army Medical Museum negative 73378.

Six cases in our series died without

significant pulmonary involvement. These cases differ from the majority and hence will be mentioned briefly. One animal died from laryngeal stenosis which followed a large abscess localized adjacent to the larynx. The disease started originally as influenza with subsequent purulent involvement of the lymph nodes of the upper respiratory tract. One animal died of purulent meningoencephalitis which reached the meninges by extension from abscesses in the pharyngeal region. Four cases developed fatal septicemia and metastatic abscesses without involvement of the lungs.

SUMMARY

A study of 150 autopsies of equine influenza as seen at a remount depot is presented. The disease is described as an acute, infectious disease of the respiratory tract with frequent complications. At the height of a severe outbreak, it assumed the characteristics of a hemorrhagic pneumonia. The etiology is complex. The disease is caused by a filterable virus associated with streptococci. The lesions in the earliest stages of the disease are confined to the turbinates, the nasal mucosa, and the lymph glands of the upper respiratory tract. Erosion of epithelium, congestion,

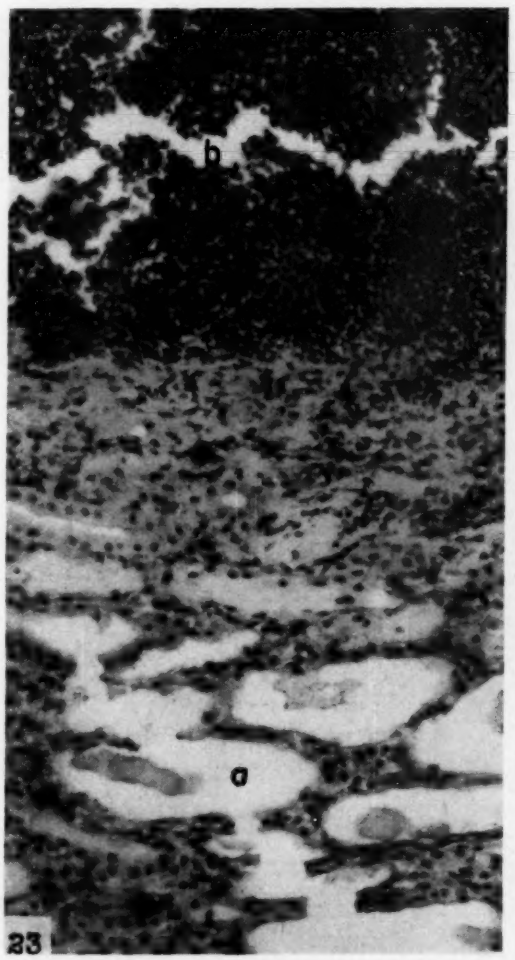


Fig. 23.—Kidney, x280. Wall of metastatic abscess. (a) Distended renal tubules, (b) abscess. Army Medical Museum negative 73420.

Fig. 24.—Cerebrum, x100. Influenzal meningoencephalitis; wall of metastatic abscess. (a) Abscess. Army Medical Museum negative 73397.

and cellular infiltration of the nasal mucosa and submucosa are probably the earliest manifestations. They are followed by marked activity and proliferation of the mucous glands. The submaxillary and parapharyngeal lymph glands are initially edematous and congested; later suppuration may occur. In the lung, the anterior and ventral parts are involved first. The initial lesions are located in the interstitial tissues and consist of edema and fibrinous infiltration, which are followed by organization and fibrosis. Consolidation of the lung tissue with hemorrhagic exudate follows the interstitial changes. The hemorrhagic exudate is later replaced by fibrinous and purulent exudates. When these exudates are not resorbed, organization with intense round cell infiltration supervenes. Liquifaction and cavitation are common complications.

The lesions in other organs are secondary and vary with the duration of the disease and the occurrence of septicemia. Abscesses in the kidney, myocardial degeneration, and passive congestion of the liver are common. Purpura hemorrhagica is an important complication. It is characterized by edema, hemorrhage, and necrosis in the subcutaneous muscles, nasal mucosa, and intestinal walls. Meningoencephalitis occasionally develops in the disease. This can be differentiated from equine encephalomyelitis by the intense purulent involvement of the meninges and the purulent nature of the perivascular exudate.

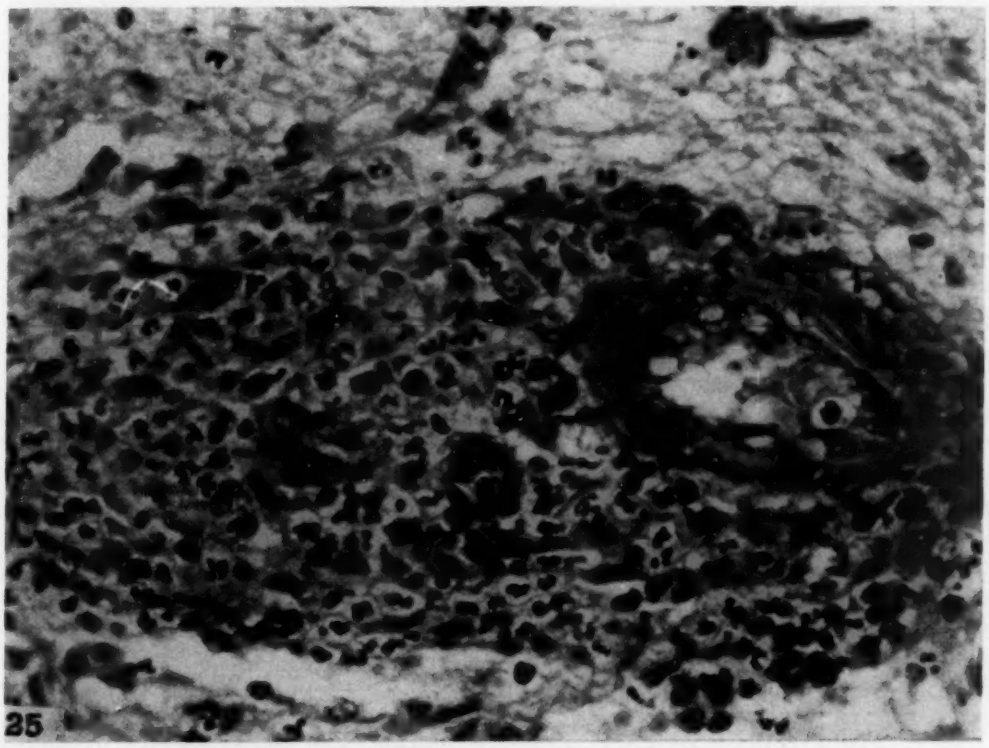


Fig. 25.— Cerebrum, x600. Influenzal meningoencephalitis. Purulent perivascular exudation. Army Medical Museum negative 73389.

ACKNOWLEDGEMENTS

The authors thank Colonel James E. Ash, Medical Corps, Curator of the Army Medical Museum, for the photographs and photomicrographs used in this paper. We also acknowledge the valuable assistance of Lieutenant Colonel Balduin Lucké, Medical Corps, who reviewed the material and assisted us in other ways.

A Superior Medium for the Hemophilic Fowl Coryza Bacillus

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SINCE THE PRIMARY isolation of the fowl coryza bacillus by DeBlieck[1] in Europe and by Nelson[2] in the United States, several investigators have reported the organism difficult to cultivate on or in artificial mediums.* The organism colonizes poorly or not at all on blood agar under ordinary incubation conditions. Nelson[2] found it necessary to seal Petri dishes with clay, and Schalm and Beach[3] could not obtain growth except when plates or slants were incubated in an atmosphere of 10 per cent carbon dioxide. Delaplane and associates[4] failed to obtain growth in blood-free mediums of several different types, and Nelson[5] stated that "it fails to grow in bouillon containing X and V factors of plant origin, suggesting that its growth requirements may not include these accessory factors."

In this laboratory, growth of the fowl coryza bacillus has been unsatisfactory until quite recently. Our tests indicate that the organism fails to colonize on blood agar unless incubated in an atmosphere of increased carbon dioxide tension, and even then, growth often fails. When seeded in blood at the base of nutrient agar slants, as suggested by Delaplane,[6] growth usually results, but is often poor. Unless the blood is fresh, growth fails to take place. Growth in this medium appears to be improved to some extent by incubating the slants in an atmosphere of 10 per cent carbon dioxide.

Published with the approval of the director as paper No. 149 of the journal series; contribution from the Department of Poultry Science, North Carolina Agricultural Experiment Station.

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While this article was in press, the writer discovered that B. H. Kessens, in a privately published doctorate thesis (Vergelijkend Onderzoek Betreffende Haemophilus Coryza, Haemophilus Influenzae en Andere Haemophiele Bacillen. Rijks Univ., Urtrecht. 1936), reported success in growing a fowl coryza organism in broth plus small pieces of potato. However, some of the cultural characteristics which Kessens ascribed to this organism strongly indicate that he was using an organism different from that studied by American workers.

In view of the poor growth obtained in and on the commonly used mediums, attempts have been made in this laboratory to develop a more satisfactory medium. A number of different mediums have been tried, among them being potato agar, potato broth, blood plus broth, filtrate from ground, uncooked chicken livers steeped in physiological saline, and chicken infusion broth plus small pieces of uncooked potato. Of the mediums tried, the blood broth and liver filtrate gave good results, and the chicken infusion plus raw potato gave excellent results.

The chicken infusion is prepared by steeping 500 Gm. of ground chicken meat in 1,000 cc. of tap water or in distilled water overnight. This is then boiled for thirty minutes, filtered through filter paper, adjusted to pH 7.5 to 7.6, autoclaved, and tubed in 5-cc. quantities. Sufficient sodium chloride is added before sterilization to make the infusion physiological. Potatoes are washed in water and then in 70 per cent alcohol. A knife is flamed and used to remove one end and two sides of a potato, the cut surfaces of which are flamed. Large pieces of potato are then removed with a sterile knife and placed in Petri dishes. The large pieces are cut into approximately 0.5 cc. squares and one square is placed in each tube of broth. The medium is incubated forty-eight hours at 37 C. before use. Thjotta and Avery,[7] in 1921, prepared a similar medium which was highly satisfactory for *Hemophilus influenzae.*

Three strains of the fowl coryza bacillus have been grown for extended periods in this laboratory in the chicken infusion-potato medium; 2 of the strains, 21 and 31, were isolated in North Carolina and the third, 44, was obtained from Dr. John P. Delaplane, now of Texas A. & M. College. At this writing, strain 21 has been consecutively transferred 19 times in chicken in-

fusion-potato broth, strain 31, 13 times, and strain 44, 14 times. Transfers have been made every five to seven days.

After twenty-four hours incubation at 37 C. in chicken infusion-potato broth, the fowl coryza bacilli strains produce a turbidity approximately equivalent to that produced by *Salmonella pullorum* in 1 per cent peptone broth in the same period. The turbidity is uniform throughout the medium and there is no pellicle produced. Further incubation usually does not produce greater turbidity. Films of cultures grown for twenty-four hours in the chicken infusion-potato broth which has been adjusted to pH 7.6 before seeding, and stained by the Gram method, show small gram-negative rods which are quite regular as to size, shape, and arrangement. The staining may be even, bipolar, or highly irregular. When strain 21 is seeded in chicken infusion-potato broth adjusted to pH 6.4, the culture tends to form long chains. All 3 strains tend to be rather pleomorphic when grown in blood at the base of nutrient agar slants.

Preliminary studies indicate that for growth to take place sodium chloride or some other salt must be present in the medium. Growth of strain 21 will take place without sodium chloride in the chicken infusion-potato medium at a pH of 7 to 7.6, provided small amounts of phosfate buffer $\left(\frac{M}{60}\right)$ are present. However, growth in such mediums tends to produce large swollen and pleomorphic forms. The data of Delaplane and associates[8] indicate that sodium chloride is essential for growth of the fowl coryza bacillus. Growth in chicken infusion-potato medium does not seem to be improved by incubation under increased carbon dioxide tension.

Koch's postulates have been fulfilled for the North Carolina strains on a number of occasions. Chickens have been infected intranasally with small quantities of 24-hour cultures in chicken infusion-potato medium. Evidence of infection is usually noted in twenty-four to seventy-two hours, and the gram-negative fowl coryza organism can usually be cultivated from the edematous facial tissue of the infected birds.

We can only speculate as to why no one has been able to obtain growth of the fowl coryza bacillus in mediums of this type long before. It is unlikely that the variety or region in which the potatoes are grown would affect the efficacy of the medium. The potatoes used in this study were grown in Idaho, Pennsylvania, and North Carolina. Potatoes from all three regions were apparently equally effective. Since the presence of sodium chloride or some other salt was found to be necessary for growth to take place, it appears possible that success was not achieved in the past because this ingredient was left out of the medium.

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Studies on Johne's Disease: Reactions to Johnin in Sensitized Guinea Pigs

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BEFORE DIAGNOSTIC agents, such as johnin and tuberculin, may be used with safety and on a large scale in the field, their potency and specificity must be assayed experimentally.

In the examination of tuberculin for these properties, the original lethal—or shock—test on tuberculous guinea pigs, originated in 1891 by R. Koch, has been modified repeatedly and a number of other biological as well as serological methods have since been tried with more or less favorable results. At present, the titration by the intracutaneous test on sensitized guinea pigs, introduced by Lewis and Aronson[15] in the United States and by Eagleton and Baxter[7] in England, is most commonly used for this purpose due to its comparative simplicity, lower cost, and fairly high accuracy. In May, 1935, the Health Committee of the League of Nations recommended this procedure with certain modifications in technique.

If tuberculin is intended for use in cattle, the results of the intracutaneous guinea pig test are usually verified on tuberculous and healthy animals of that species before issue.

The proper standardization of johnin by similar methods presents some difficulties not encountered with tuberculin for the reason that laboratory animals are refractory to Johne's disease, and naturally or artificially infected cattle show during the course of the disease a varying degree of sensitivity toward the specific diagnostic agent. Such fluctuations in the allergic state have been observed by Minett[17] during periodic intradermal johnin tests in natural cases while similar results were obtained in this institute in artificially and naturally infected cattle.

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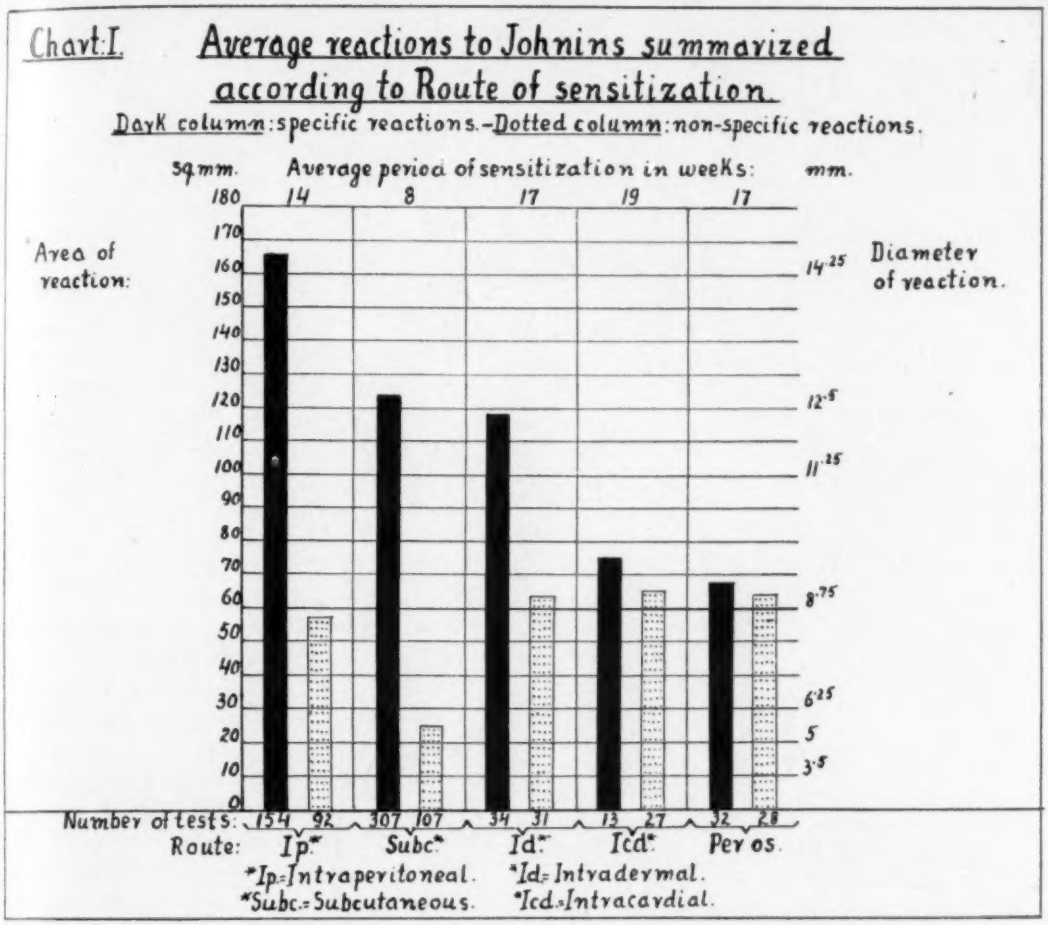
In the absence of a suitable laboratory animal, the bovine species has been used generally for an approximate evaluation of johnins, before their issue to the field. Vallée and Rinjard[20, 26] checked the potency of their product on cattle, artificially sensitized by subcutaneous inoculation of Johne's bacilli suspended in mineral oil to which was added some inert substance, such as powdered glass or sand. Dunkin[6] claimed good results in the assaying of the specific and nonspecific properties of successive lots of johnins by intradermal tests on healthy and naturally infected cattle. Buxton and Glover[2] attempted to increase the sensitivity of cattle to johnin by subcutaneous injections of Johne's bacilli coated with gum acacia and suspended in mineral oil. A marked allergy was attained which soon declined in most of the treated animals.

As the maintenance of even a small number of cattle infected with Johne's disease is expensive and their housing and isolation cumbersome, the use of small, properly sensitized, laboratory animals would obviously have many advantages in the standardization of johnin.

That guinea pigs acquire a certain degree of sensitivity toward johnin after the inoculation of large doses of Johne's bacilli has been known for many years. Boquet[1] was one of the first to study the allergic reactions of these animals after the intraperitoneal injection of 100 to 150 mg. of living cultures of Johne's bacilli. After a lapse of fifteen to twenty days the guinea pigs responded to the intradermal injection of 0.1 cc. johnin in a dilution of 1 : 10 with a well marked edematous swelling appearing toward the eighteenth hour and increasing in size up to the forty-eighth hour. Generally, no necrosis was observed. The reactivity of the animals persisted for more



than eight months. An even more extended local reaction, often progressing to necrosis, was obtained in the same guinea pigs to similar doses of avian and bovine tuberculin.

Hagan and Zeissig[11] used for the sensitization of guinea pigs heavy suspensions of Johne's bacilli in paraffin oil applied subcutaneously. The sensitization of their animals became apparent approximately twenty-one days after the injection of the bacillary suspension and the reactions were well marked. While examining the comparative value of avian tuberculin and johnin in intradermal tests on the sensitized guinea pigs, these workers observed that some of their johnin batches were of lower potency than the avian tuberculin of apparently standard strength.

After completion of this paper, the articles of Glover[10] and of Johnson and Cox[13] were published. Glover used guinea pigs, sensitized by intraperitoneal injections of Johne's bacilli suspended in mineral oil, in extensive intradermic standardization tests of heat-concentrated johnins as well as of their purified protein derivative (p.p.d.). The sensitization in successive batches of guinea pigs appeared to be of a similar level and permitted the determination of a 50 per cent variation in potency as compared with a johnin standard chosen at random.

Johnson and Cox, however, were unable to obtain a lasting and uniform sensitization of guinea pigs with either subcutaneous or intraperitoneal injections of Johne's bacilli suspended in mineral oil. In their experiments, male birds of the White Leg-

horn breed proved to be more suitable for the standardization of johnin.

Since June, 1935, experiments have been under way in the Animal Diseases Research Institute with the object of determining the usefulness of guinea pigs for the biological evaluation of the johnins prepared in the Institute. At this time, preliminary intradermal johnin tests made on animals inoculated subcutaneously and intraperitoneally with doses varying from 5 mg. to 30 mg. for the primary purpose of determining the purity of the Johne's bacillus stock cultures, showed that guinea pigs so treated became sensitized to johnin. As the reactivity of the animals varied to a considerable extent according to the route of injection and the time elapsed since the administration of the bacilli, it seemed of foremost importance to determine more closely:

1) The relationship of the route of inoculation to sensitization.

2) The time period at which the peak of sensitization is reached.

The first part of the work was, therefore, concerned mainly with the gathering of experimental data in respect to these two factors. Numerous guinea pigs, divided into smaller or larger groups, were inoculated by various routes with a single dose of living cultures of Johne's bacilli and tested at more or less regular intervals with johnins prepared in this laboratory.

Five routes of sensitization were compared, directing the main attention to the intraperitoneal and subcutaneous methods. The doses of Johne's bacilli injected varied to some extent according to the route, but were as a whole, closely comparable:

a) Intraperitoneal route	:	dose 30 mg.
b) Subcutaneous route	:	dose 50 mg.
c) Intradermal route	:	dose 30 to 50 mg.
d) Intracardial route	:	dose 10 to 20 mg.
e) *Per os* route	:	dose 30 to 50 mg.

Adopting the vehicle used by Calmette and Guérin in the vaccination of calves with BCG. against tuberculosis,[14] the Johne's bacilli were suspended in a mixture of one part of Sauton's synthetic medium and three parts of physiological saline solution, which henceforth will be referred to as Sauton saline. However, in one series of experiments carried out with the subcutaneous route, paraffin oil was used parallel with Sauton saline as a vehicle for the bacillary suspension. In consequence, approximately 60 per cent of the johnin tests summarized in chart 1 under "subcutaneous route" refer to tests made on guinea pigs sensitized by this modified method.

The amount of johnin injected remained invariably 0.1 cc. Various preparations were applied but they may be divided into two main groups:

a) Concentrated to 1/10 original volume in the usual manner on the water bath.

b) Purified and concentrated by precipitation with ammonium-sulfate, or by ultrafiltration, or by ultrafiltration followed up with trichloracetic acid precipitation (p.p.d.), respectively.

While the products obtained by ammonium-sulfate-precipitation or ultrafiltration were applied in a concentration similar to that of the heat-concentrated johnin, the p.p.d. preparations, used in a dilution of 1 : 500, represent a higher concentration.

In the experiments, examining the effect of the five different routes of sensitization, roughly speaking, a similar number of tests were carried out with heat-concentrated products, and with products purified by ammonium-sulfate precipitation or by ultrafiltration. In the subcutaneous group, p.p.d. was used in addition to johnins purified by precipitation or ultrafiltration, augmenting the number of tests made with this group of johnins to 90 per cent of the total. This fact should be kept in mind, when conclusions are drawn from the results analyzed in the charts 1, 2 and 3.

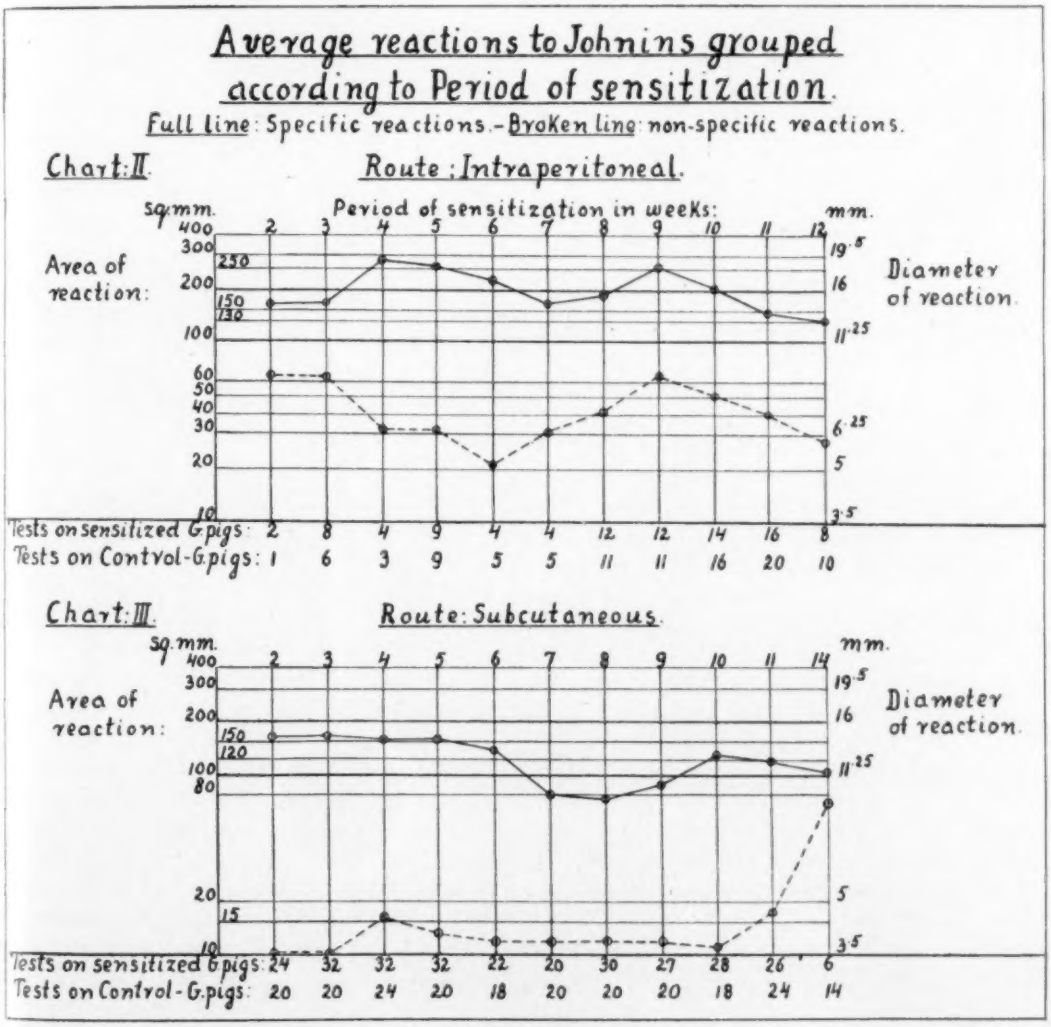
The readings were made at the twenty-fourth, forty-eighth and seventy-second hours but the 48-hour results have been used in the final compilation, as the reactions appeared to be most pronounced at this period. Each reading included the registration of the area covered by the skin reaction, the thickness of the edematous swelling, the intensity of redness and the area of infiltration and necrosis, if the latter occurred during the period of observation.

The area covered by the skin reaction appeared to parallel closely the other signs of reaction and could be determined with fair accuracy from the diameter readings taken by a caliper. These readings, therefore, were chosen as a guide in the deterination of the degree of reaction, and the calculated averages, expressed in square millimeters, were plotted on the ordinate of the coördinate system in all the charts analysing the results.

1) In order to answer in a general way the question regarding the comparative effectiveness of the various routes of inoculation in the sensitization of guinea pigs, the average response was calculated for each group separately.

To this end, all 48-hour readings were summarized and averaged according to the route used for sensitization, irrespective of the period which had elapsed since the sensitizing dose was given. A similar procedure was followed in the compilation of the corresponding nonspecific reactions observed in normal guinea pigs. In chart 1, the figures expressing the average areas in square millimeters for each of the 5 groups are plotted in serial order on the ordinate of the coördinate system. The number of tests on which the averages are based, as well as the average period of sensitization which they represent, is indicated on the abscissa.

By subtracting the nonspecific value, ob-



tained in normal guinea pigs, from the corresponding specific value, the clear specific antigenic response is obtained for each group.

Proceeding in such manner in the interpretation of the results summarized in chart 1, it becomes evident that the intradermal, intracardial and *per os* route are of little or no value for the sensitization of guinea pigs. After the intraperitoneal and subcutaneous injections, more pronounced reactions of almost identical strength are obtained. The fact that approximately 60 per cent of the tests compiled in the subcutaneous group refer to guinea pigs sensitized by the use of parafin oil as a vehicle for the bacillary suspensions, and that almost 90 per cent of the tests were carried out with purified johnin preparations of partly higher concentration and low nonspecific properties suggests, however, a superiority of the intraperitoneal route above the subcutaneous, should identical experimental conditions prevail.

It may be noted from the data in chart 1 that the average period of sensitization serving as a basis of comparison varies in the five groups. The conclusions drawn from chart 1 may be verified if strictly corresponding reactions are compared. For this purpose, the allergic response obtained in weekly intervals after the use of the different modes of inoculation has been plotted on semilogarithmic grids. Charts 2 and 3 represent such a detailed analysis of the reactions obtained from the second to fourteenth week in the intraperitoneal and subcutaneous group given in chart 1.

2) The closer determination of the time period during which the peak of reactivity in the treated animals is reached appears to be of practical value during routine potency tests, especially for the proper timing of the sensitizing dose and of the subsequent standardization experiments.

A sufficient number of tests for the determination of the clear-cut trend of the reactivity over an extended time period were available only in the groups dealing with the intraperitoneal and subcutaneous method of sensitization. The intradermal, intracardial and *per os* routes, have, in general, given only few pronounced reactions. Charts 2 and 3 give the information in regard to the reactivity of guinea pigs at various time periods after sensitization by the intraperitoneal and subcutaneous methods, respectively. The sums of the control tests in these charts appear higher than in chart 1, due to the fact that, depending on the set-up of the experiments, often the same tests on healthy animals controlled the specific reactions on sensitized guinea pigs obtained in several consecutive weekly intervals.

The trend of the curves in charts 2 and 3 shows that the reactivity of the sensitized guinea pigs is already high at the second or third week after injection of the bacillary culture.

While the sensitization in the subcutaneous group remains on approximately equal levels to the sixth week, it varies after intraperitoneal injection in irregular fashion to the tenth week. After these periods, definitely lower averages are registered.

Thus, the foregoing survey of experiments, concerned mainly with the general question of effective sensitization of guinea pigs to johnin has given favorable results, especially if the bacillary suspensions are introduced by the intraperitoneal and subcutaneous route. It was noted, however, that the allergy elicited in the various groups of guinea pigs occasionally was irregular in intensity and duration.

In subsequent trials, it was attempted to reinforce and prolong the reactivity of the guinea pigs by rendering them more susceptible to the action of the injected specific organisms.

Thomson[25] and Hagan and Levine[12] found that living saprophytic acid-fast or dead tubercle bacilli, if suspended in paraffin oil and introduced subcutaneously or intramuscularly, will produce metastatic lesions in the adjacent lymph glands and the lungs of guinea pigs which resemble those caused by virulent tubercle bacilli. Similar observations were made by Rist[21] after the subcutaneous inoculation of dead tubercle bacilli incorporated in paraffin or paraffin oil.

For the easier demonstration of the fil-

trable forms of tubercle bacilli, van Deinse[5] introduced the material under investigation into the abdominal cavity of guinea pigs previously treated intraperitoneally with Ca-phosfate. In the small droplets of pus formed on the peritoneum and omentum as a reaction to the Ca-phosfate, the filtrable forms found favorable conditions for growth and multiplication.

Assuming that such action of the Ca-salt on the peritoneum also may promote the susceptibility of guinea pigs to other microörganisms, Sedlmeier[24] used the same technique with success in the experimental transmission of actinomycosis.

The work of Coulaud[4], Saenz[22, 23] and Freund, Casals and Hosmer[8] have shown that paraffin and paraffin oil, when used as vehicles for the suspension of dead tubercle bacilli, greatly enhance the allergic power of these organisms in guinea pigs and rabbits, producing a durable and greatly increased sensitization of these animals to tuberculin.

It is evident from these reports that the use of various vehicles for the treatment of guinea pigs before inoculation, or for the suspension of otherwise harmless microörganisms, changes not only the pathogenic action of these organisms but produces also a higher allergic response in the treated animals.

The adoption of similar methods for a more forceful sensitization of guinea pigs to johnin seemed to be the more warranted, as good results in this respect already had been attained by Hagan and Zeissig,[11] in guinea pigs, and by Vallée and Rinjard[20, 26] in cattle. For our particular purpose, two of the above named procedures were adopted: *a*) the use of paraffin oil as a vehicle for the preparation of the bacillary suspensions, *b*) the Ca-phosfate treatment of van Deinse prior to the intraperitoneal inoculation of the Johne's bacilli suspended in Sauton saline.

Both methods were applied on large numbers of guinea pigs injecting the animals by the intraperitoneal route. In order to obtain an even distribution of the bacilli in paraffin oil, a dense suspension was first prepared in Sauton saline, adding this to the sterile paraffin oil in such proportion that a 50 to 75 per cent final oil content of the vehicle resulted.

The Ca-phosfate treatment of van Deinse is carried out in the following manner: 5 per cent solutions of $CaCl_2$ (6 H_2O) and Na_2HPO_4 (12 H_2O) are sterilized and mixed in a ratio of 1 part $CaCl_2$ to 4 parts Na_2HPO_4. After thorough shaking of the resulting heavy white precipitate, 2.5 cc. are injected intraperitoneally into each guinea pig; forty-eight hours later the sensitizing dose of Johne's bacilli suspended in Sauton saline is introduced by the same route.

Concurrently, parallel series of guinea pigs were sensitized by the intraperitoneal route with suspensions of Johne's bacilli in Sauton saline without previous treatment with Ca-phosfate.

The guinea pigs of the three experimental groups, representing three modes of intraperitoneal sensitization, were subdivided into three or four batches and johnin tests were conducted in rotating, weekly intervals. In this manner the johnin test on the individual animal was repeated only every third or fourth week. In order to eliminate all variable factors as far as possible, the sensitizing dose was kept at 15 mg., prepared by pooling 6 strains of Johne's bacilli in equal quantities of moist weight; furthermore, only johnin p.p.d. preparations were applied throughout, in a dilution of 1 : 500 and in a dose of 0.1 cc.

The subcutaneous route of sensitization with Sauton saline and mineral oil as respective vehicles was employed under identical experimental conditions, except that the sensitizing dose of Johne's bacilli was raised to 50 mg. moist weight. Sufficient tests with p.p.d. johnins on animals sensitized by the above subcutaneous methods were conducted during the preliminary trials, compiled in charts 1 and 3, to make additional experiments on this line superfluous. These tests were now rearranged for comparison with the corresponding tests of the groups, sensitized intraperitoneally.

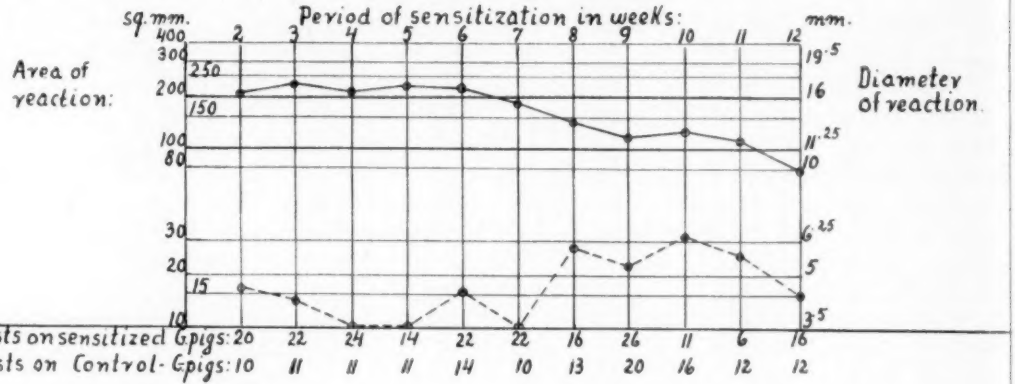
The results were summarized in five semilogarithmic grids (4 to 8) which rep-

Average reactions to P.P.D.-Johnins grouped according to Period of sensitization.

Route: Intraperitoneal.

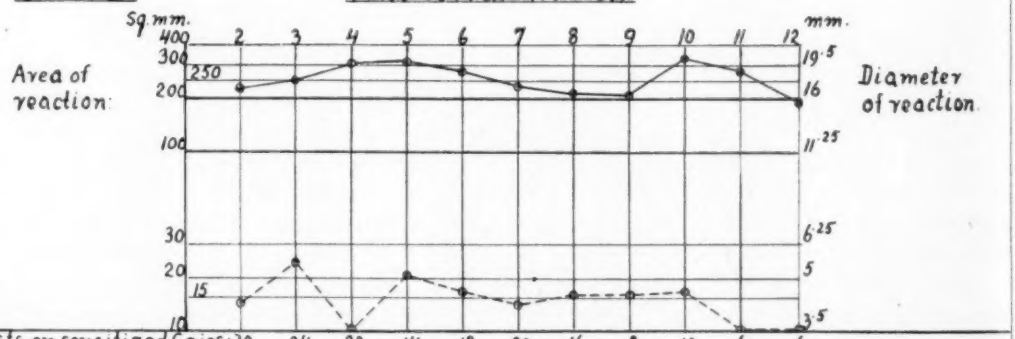
Full line: Specific reactions. Broken line: Non-specific reactions.

Chart: IV. Vehicle: Sauton-saline.



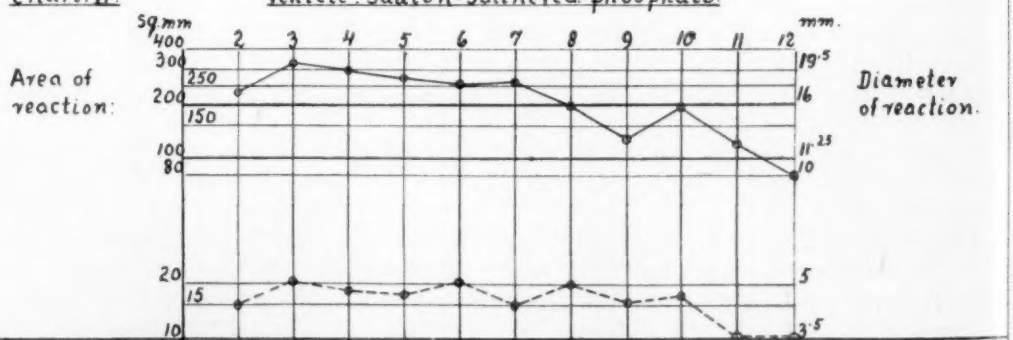
Tests on sensitized G.pigs:	20	22	24	14	22	22	16	26	11	6	16
Tests on Control-G.pigs:	10	11	11	11	14	10	13	20	16	12	12

Chart: V. Vehicle: Paraffin-oil.



Tests on sensitized G.pigs:	30	24	22	14	18	20	16	8	10	6	6
Tests on Control-G.pigs:	18	14	20	10	16	14	20	14	10	10	8

Chart: VI. Vehicle: Sauton-saline+Ca-phosphate.



Tests on sensitized G.pigs:	65	66	67	29	44	40	33	42	20	18	14
Tests on Control-G.pigs:	35	35	36	19	25	18	29	24	10	10	8

resent reaction curves obtained under the following experimental conditions:

Chart 4.—Intraperitoneal sensitization with Johne's bacilli suspended in Sauton saline.

Chart 5.—Intraperitoneal sensitization with Johne's bacilli suspended in paraffin oil.

Chart 6.—Intraperitoneal sensitization with Johne's bacilli suspended in Sauton saline preceded by the treatment with Ca-phosfate.

Chart 7.—Subcutaneous sensitization with Johne's bacilli suspended in Sauton saline.

Chart 8.—Subcutaneous sensitization with Johne's bacilli suspended in paraffin oil.

When the antigenic effect of Johne's bacilli suspended in Sauton saline and applied intraperitoneally or subcutaneously is compared as a whole with that of the paraffin-oil suspensions, introduced by corresponding routes, a decided inferiority of the former becomes apparent. (Charts 4 and 7 against charts 5 and 8.)

When, on the other hand, the reactions obtained after subcutaneous sensitization as such are measured against those obtained after the intraperitoneal sensitization, the subcutaneous route proves to be of lower efficiency. (Charts 7 and 8 against charts 4, 5 and 6.)

Conclusive deductions from the reaction curves, presented by the various experimental groups, may be made most conveniently if the reaction of 16 mm. diameter is chosen as an arbitrary standard.

After subcutaneous sensitization with the Sauton saline suspension of Johne's bacilli (chart 7), this level is not reached at any time, while the intraperitoneal injection of such suspensions raises the reactions to closely above this standard during a period extending from the second to the sixth week, with a gradual decline hereafter (chart 4).

The paraffin-oil suspension applied subcutaneously (chart 8) has about the same effect as the Sauton saline suspension given intraperitoneally (chart 4).

The paraffin-oil suspension introduced into the abdominal cavity (chart 5) produces a curve sustained well above 200 sq. mm. for eleven weeks with two peaks at the fourth, fifth, and the tenth week, respectively.

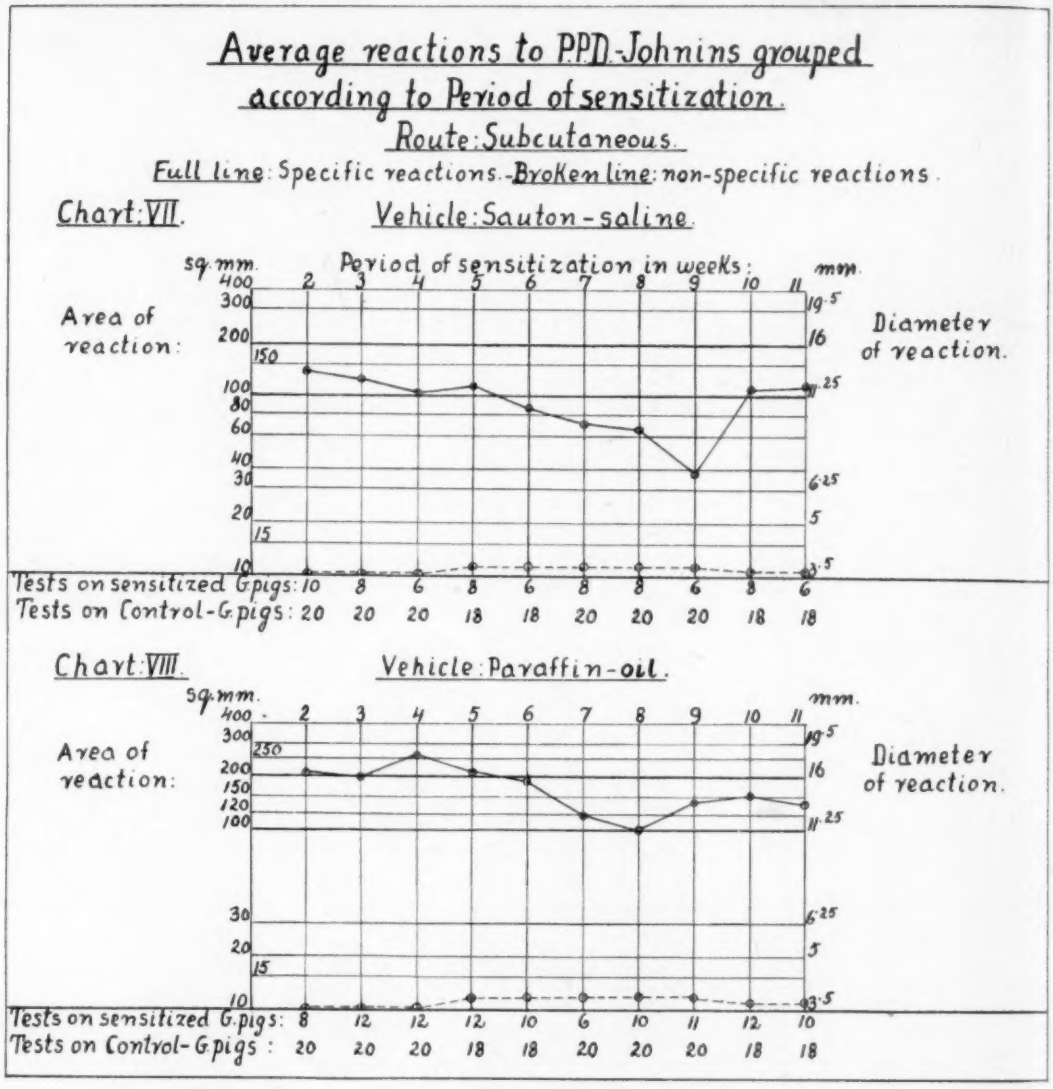
Comparing, finally, the results of the Ca-phosfate method of van Deinse (chart 6) with those obtained with the highly effective, intraperitoneal paraffin-oil technique (chart 5), the reactions appear closely comparable in the first eight weeks. From the ninth to twelfth week, the reactivity of the guinea pigs treated with Ca-phosfate drops slightly below the level obtained in the series sensitized with paraffin-oil suspensions.

DISCUSSION

The reactivity to johnin of guinea pigs sensitized in various ways by the inoculation of living cultures of Johne's bacilli was examined in approximately 1,900 intradermal tests, including over 600 control tests. While several variable factors existed in the preliminary general survey, an approximation of the reaction trend was, nevertheless, obtained.

In subsequent tests, which were designed to verify the primary findings and to examine the usefulness of various vehicles for a more lasting and pronounced sensitization, stable experimental conditions were maintained as far as possible. The final results have shown that the intraperitoneal route is the most effective one for the sensitization of guinea pigs, and that the use of paraffin oil as a vehicle or the intraperitoneal treatment of the animals with Ca-phosfate prior to the injection of the bacillary suspension in Sauton saline by the same route enhances the degree and duration of reactivity to a considerable extent.

The comparison of the reaction curves shown in charts 5 and 6 indicates that the sensitization elicited by the Ca-phosfate method is of similar strength but of somewhat shorter duration than that produced by the use of paraffin oil as a vehicle for the bacilli. On the other hand, fewer complications in the treated animals have been observed after treatment of the animals with Ca-phosfate and it has, therefore, proved more satisfactory in our hands. Only a few guinea pigs died during the first three weeks after inoculation showing more or less pronounced hyperemia of the peritoneum, occasionally signs of liver degeneration, swelling of the spleen, hyperemia


Average reactions to P.P.D.-Johnins grouped according to Period of sensitization.
Route: Subcutaneous.
Full line: Specific reactions. Broken line: non-specific reactions.
Chart: VII.
Vehicle: Sauton-saline.
sq. mm.
Period of sensitization in weeks:
mm.
Area of reaction:
Diameter of reaction.
400 300 200 150 100 80 60 40 30 20 15 10
2 3 4 5 6 7 8 9 10 11
19·5 16 11·25 6·25 5 3·5
Tests on sensitized G.pigs: 10 8 6 8 6 8 8 6 8 6
Tests on Control-G.pigs: 20 20 20 18 18 20 20 20 18 18
Chart: VIII.
Vehicle: Paraffin-oil.
sq. mm.
mm.
Area of reaction:
Diameter of reaction.
400 300 250 200 150 120 100 30 20 15 10
2 3 4 5 6 7 8 9 10 11
19·5 16 11·25 6·25 5 3·5
Tests on sensitized G.pigs: 8 12 12 12 10 6 10 11 12 10
Tests on Control-G.pigs: 20 20 20 18 18 20 20 20 18 18

and edema of the lungs. The mortality was negligible if healthy animals of not less than 700 to 800 Gm. weight were chosen. In the later periods of sensitization (between the seventh and tenth week) generally some additional animals gradually lost weight due to slowly developing adhesions between various abdominal organs and the peritoneum, as well as progressive degeneration of the liver terminating in death from some intercurrent disease. These complications did not interfere with the standardization tests, as they left the animals in fairly good physical condition and with unimpaired reactivity during the period of highest sensitization.

The resensitization of surviving experimental animals with Johne's bacilli, even if applied after an elapse of five to six months, proved to be impracticable and resulted in great losses twenty-four to forty-eight hours after the reinoculation. The most pronounced and most frequently observed lesions were severe, often exudative peritonitis with hemorrhagic infiltration around the localized lesions, set up by the previous treatment. This reaction may well be compared with that encountered in tuberculous

animals during titration of tuberculin by the shock test.

If the Johne's bacilli were suspended in a vehicle containing 75 per cent paraffin oil, the intraperitoneal injection caused the death of a large portion of the animals before they could be used in titration. Exudative pleurisy and peritonitis with adhesions of fibrinous and later fibrous character were mainly responsible for the loss.

The reduction of the oil content of the vehicle to 50 per cent lowered the mortality rate, but emaciation of many animals as early as six to seven weeks after injection disturbed the development of a proper sensitivity and characteristic reactions to johnin have not been often encountered.

According to the results summarized in charts 4 to 8 the use of either Ca-phosfate or paraffin oil in conjunction with living cultures of Johne's bacilli, applied intraperitoneally, has raised not only the level and duration of sensitivity, but also attained these results by the injection of a comparatively small, single dose of Johne's bacilli, simplifying the technique of sensitization.

In attempts to determine the comparative potency of various lots of johnin p.p.d. on sensitized guinea pigs, the intradermal test has been tried in the manner described by Calmette and De Potter,[3] by Okell and Parish[19] and others, for the titration of tuberculin and adopted for this purpose with modifications in technique by the Health Committee of the League of Nations in May, 1935. The results obtained in the standardization of various batches of johnin p.p.d., prepared in this institute, will be reported later. In the course of these tests, it was found that the strongest concentration of johnin p.p.d. not eliciting a nonspecific reaction in normal guinea pigs, was generally in the neighborhood of 1:500 if applied in 0.1 cc. doses. In this strength, none of our preparations has given rise to swelling in known healthy cattle.

From the great volume of experimental evidence available on standardization and the practical application of tuberculin in the eradication of bovine tuberculosis, the dangers connected with the use of a highly concentrated product have been recognized.[9] But it also became established that preparations of low potency will miss a percentage of affected animals. The results of extensive comparative herd tests conducted in England under the supervision of Montgomerie and Thomson[18] with tuberculins of different origin and varying concentration have clearly illustrated this point.

It is generally agreed that johnin is not as efficient in detecting Johne's disease as tuberculin for the diagnosis of tuberculosis in cattle. The need for a potent johnin used in the highest concentration, which will elicit no nonspecific reactions in healthy animals, is evident.

By the isolation of the p.p.d. from johnin, the nonspecific properties of this product have been largely eliminated and the potency of the various lots were found to be closely comparable.

The use of such preparations in dilutions of 1 : 500 or even 1 : 400 in tests conducted under governmental supervision for the elimination of Johne's disease from infected herds has yielded promising results.[16] In the light of these experiences, it appears to indicate the application of the johnin p.p.d. in such concentrated form to cattle, provided the standardization tests in guinea pigs have proven that the product under examination is equal or closely comparable in its specific and nonspecific properties to the chosen standard.

CONCLUSIONS

1) The relative effect of various routes and vehicles in the sensitization of guinea pigs to johnin by the injection of Johne's bacilli have been examined in approximately 1,900 intradermal tests with johnins prepared in this institute.

2) The intraperitoneal application of a comparatively small dose of Johne's bacilli in animals treated forty-eight hours previously with a suspension of Ca-phosfate introduced by the same route, has given the most favorable results.

3) Guinea pigs sensitized by this method were used with success for potency-determinations of johnin p.p.d. in intradermal

tests carried out along the lines followed in the standardization of tuberculin.

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Effect of Injections of Novoxil on the Bovine Udder Infected with Mastitis Streptococci

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Madison, Wisconsin

THE INCREASED use of injections of various antiseptics in the udder for the treatment of bovine mastitis has prompted this study to determine the effect of one of these materials on the quality of the milk and on the pathological changes in the udder. Klein, *et al.*,[1] reported that Novoxil-treated quarters returned to the pretreatment condition in five to thirteen days as determined by physical examination. Weirether, *et al.*,[2] found high leucocyte counts during injections with silver oxide and, in some quarters, for many days afterward. Little, *et al.*,[3, 4] state that the foremilk from quarters treated with Novoxil contained a large amount of débris which in some cases persisted for nearly a week. They also reported high pH, chlorides, and cell counts.

TREATMENT OF LACTATING QUARTERS

The animals selected for treatment during lactation were 2 aged cows with a history of recurrent mastitis. By a physical examination, fibrosis was found in the right hind quarter of cow 146 and in all 4 quarters of cow 58. Before treatment was begun, 2 series of quarter milk samples were drawn aseptically and subjected to a Hotis test,[5] titration for chlorides with the method of Rosell,[6] a catalase test using the Johns and Hastings[7] tube, and dilution plating in veal infusion agar containing 7 per cent of bovine blood. Streptococci isolated from 5 quarters were given a biological examination in various culture mediums and found to conform to Little's[4] characters for *Streptococcus agalactiae*. The right hind quarter of cow 146 and the 2 right quarters of cow 58 were injected 3 times, at intervals of seventy-two hours, with 10 cc. of Novoxil.* This product contains 5 per cent of silver ovide suspended in mineral oil. The quarters were thoroughly milked out before injection, and the material was left in the glands until the following milking twelve hours later.

The cows were examined every day for evidences of inflammation, and quarter samples were drawn every three days, just before the next injection, to be subjected to the tests indicated above. Later, the samples were drawn less often. Sixteen days after the last injection, the cows were slaughtered at a local abattoir and the udders collected for gross pathologic examination. Small pieces of tissue from various parts of each quarter were fixed in Zenker's solution, sectioned, and stained with hematoxylin and eosin. The stained sections were then examined for histopathological changes.

Published with the approval of the research director of the Wisconsin Agricultural Experiment Station.

From the Department of Veterinary Science, University of Wisconsin.

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[2]Weirether, F. J., Anderson, E. O., Johnson, R. E., Plastridge, W. N., and Jungherr, E.: A Preliminary Report on the Effect of Colloidal Silver Oxide on Bovine Mastitis. *Ibid.*, pp. 141-144.

[3]Little, R. B., Dubos, R. J., and Hotchkiss, R. D.: Gramicidin, Novoxil, and Acriflavine for the Treatment of the Chronic Form of Streptococcic Mastitis. J.A.V.M.A., *98* (Mar., 1941), pp. 189-199.

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[5]Hotis, R. P., and Miller, W. T.: A Simple Method for Detecting Mastitis Streptococci in Milk. U. S. Dept. Agric., Circ. 400, (Aug., 1936), pp. 1-6.

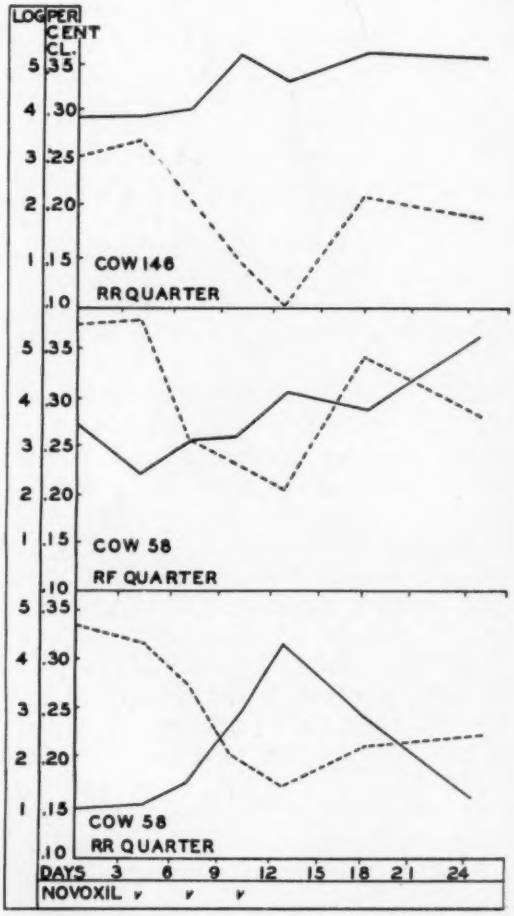
[6]Rosell, J. M.: Some Practical Conclusions from Many Years of Mastitis Studies in European and North American Countries. Can. Ayrshire Rev., *17* (June, 1936), pp. 78-81, 100-103.

[7]Johns, C. K., and Hastings, E. G.: Concerning the Use of Indirect Biochemical Tests for the Diagnosis of Chronic Bovine Mastitis. Can. J. Research, D, *16* (1938), pp. 6-14.

*The supply of Novoxil for this study was made available to the Department of Veterinary Science by E. R. Squibb and Sons.

RESULTS

As compared with the 2 infected and the 3 noninfected quarters which were left untreated as controls, the treated lactating quarters showed an acute inflamatory reaction beginning within twenty-four hours after the first injection. The affected quarters were hot, swollen, and painful, and the



GRAPH 1.—The number of organisms and the amount of chlorides in the secretion of 3 lactating quarters injected with 10 cc. of Novoxil on the fourth, seventh, and tenth days.

.....Percentage of soluble chloride

.....Logarithm of the plate count of streptococci per cc.

secretion from each quarter showed a marked increase in chloride content. (See graph 1.) While the physical evidences of inflammation (redness, heat, swelling, and pain) were reduced by the forty-eighth hour and nearly gone by the seventy-second hour, the percentage of chlorides continued to increase during the injections, and remained high in 2 of the 3 quarters until the cows were slaughtered. The catalase content increased during treatment, but returned to the pretreatment level by the time of slaughter. In all of the quarters, the number of streptococci per cc. of milk was reduced markedly by treatment, and no organisms were found in the right hind quarter of 146 three days after the last injection. However, on later tests, streptococci were found in the secretion of all 3 quarters. A significant difference in the reaction of the quarters was the apparent return to near the normal state of lactation by the right hind quarer of 58, which was normal before treatment, while the right fore of 58 and the right hind of 146, which were markedly abnormal before treatment, were more abnormal afterward.

A puzzling phenomenon was the negative Hotis reaction observed following treatment once in the right hind quarter of 58, and 5 times in the right hind of 146, even though viable streptococci were found by plating and subsequent cultural technic. A possible explanation might be the oligodynamic action of minute amounts of ionized silver as described by McCulloch.[8] A more plausible answer is that the small number of organisms eliminated were unable to establish themselves in the Hotis tube when the abnormal secretion was mixed with normal, sterile milk, as described by Pounden.[9]

Gross inflammatory changes were observed in all 3 quarters on postmortem examination. Several small chronic abscesses were present in the right hind quarter of cow 146 and both this quarter and the right fore quarter of cow 58 were dark brown on cross section in contrast to the whitish-pink color observed in the control quarters. An interesting gross feature was a distinct greyish discoloration apparently produced by a retention of particles of silver oxide. This greyish discoloration

[8]McCulloch, E. C.: Disinfection and Sterilization. Lee and Febiger, Philadelphia (1936), pp. 235-236.

[9]Pounden, W. D.: Deferred Milking—A Method of Handling Mastitis. Cornell Vet., *31* (Oct., 1941), pp. 339-344.

was most abundant in the ventral part of the quarters, but some was present in the dorsal one-third of the secreting tissue.

The histopathological lesion common to all 3 treated quarters was the presence of a recent, acute inflammatory process superimposed upon chronic changes of mastitis. Foreign-body giant cells containing a dark yellowish-brown material were observed adjacent to the alveoli and collecting ducts. These giant cells were not observed in sections from the untreated quarters, so they may be a reaction to the presence of microscopic flakes of retained silver oxide.

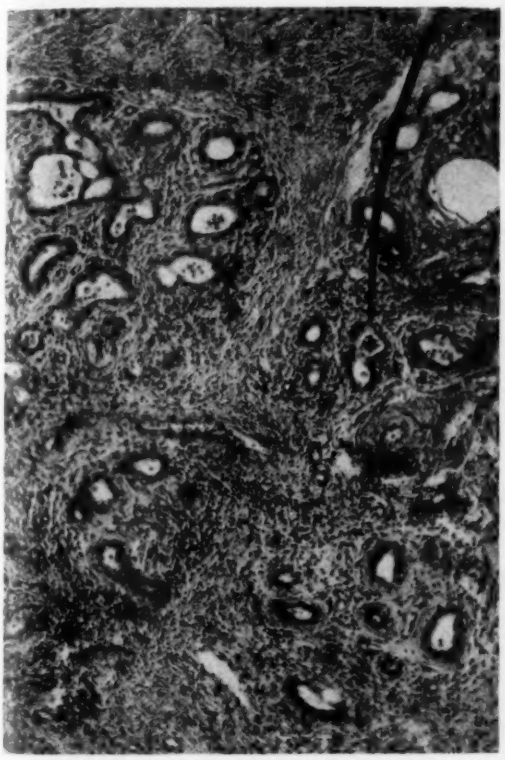


Fig. 1.—Right hind quarter of cow 146, showing chronic fibrosis with a recent acute inflammatory process superimposed. At the end of the pointer is an alveolus containing polymorphonuclear leucocytes. x100.

The treated right hind quarter of cow 146 was found to have the gross and histopathological lesions of chronic purulent mastitis in most sections examined. However, in other areas a relative increase in the amount of connective tissue and an infiltration into the tissue of lymphocytes characteristic of interstitial mastitis was observed. An exudate of polymorphonuclear leucocytes and lymphocytes appeared in most of the patent ducts and alveoli. The epithelium lining the larger collecting ducts showed proliferation and in places folds and papillae were observed.

Sections from the treated right fore quarter of cow 58 were found to have acute, severe inflammatory changes, such as exudation of polymorphonuclear leucocytes into the alveolar lumen and congestion. In addition to the acute changes, the alveoli in some areas were partially collapsed or atrophied, giving the appearance of a relative increase of connective tissue. The sections of the treated right hind quarter of cow 58 showed some exudation of

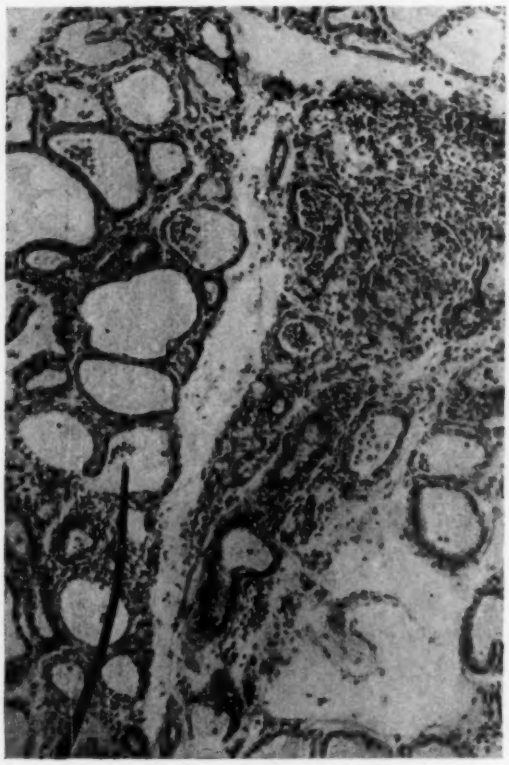


Fig. 2.—Right fore quarter of cow 58. Both acute and chronic changes are present, but the acute changes are more prominent in this section. The alveolar exudate at the end of the pointer is mainly polymorphonuclear leucocytes. x100.

polymorphonuclear leucocytes into the alveolar lumen, but in much less degree than the right fore quarter. In contrast to the acute inflammatory changes observed in the 3 treated quarters, the untreated left hind quarter of cow 58 (which was eliminating from 30,000 to 50,000 *Str. agalactiae* per cc. and gave abnormal amounts of chlorides on about one-half of the tests) showed only the chronic changes of increased connective tissue in the interalveolar and interlobular spaces and a slight infiltration of lymphocytes between the alveoli. In both the treated and untreated quarters, the ventral portion of each quarter was found to be more abnormal than the dorsal portion. This agrees with the findings of Morrill.[10]

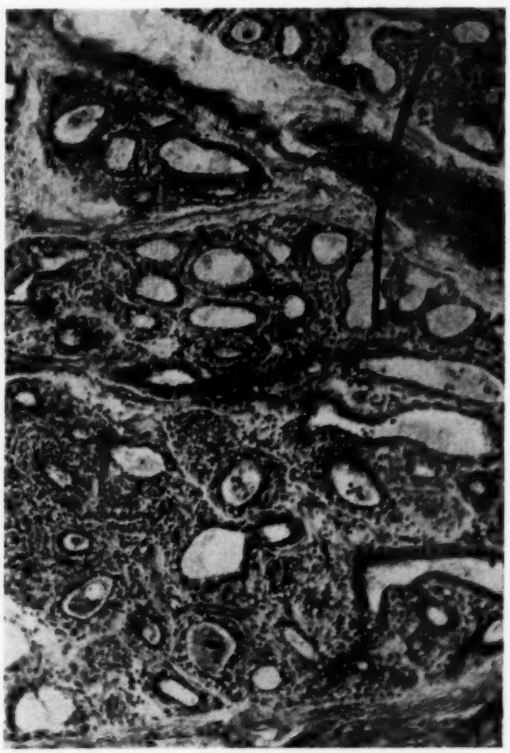


Fig. 3.—Right hind quarter of cow 58. The most prominent change here is chronic interstitial mastitis with infiltration of lymphocytes and monocytes. x100.

[10]Morrill, C. C.: A Histopathological Study of the Bovine Udder. Cornell Vet., 28 (1938), pp. 196-210.

TREATMENT OF DRY QUARTERS

Twenty-one quarters of 6 cows with a history of recurrent mastitis were injected with 10 cc. of Novoxil when in the dry period. Streptococci had been isolated from the quarters at the end of the previous lactation period, and cultural tests indicated they were mastitis streptococci. All the quarters were in a quiescent state except the left fore quarter of M2, which had a severe acute catarrhal galactophoritis at the time of injection. The material was left in the quarters till the colostrum was removed after calving.

A slight to moderate swelling following injection was observed in all quarters, and the quarter with acute inflammation of the lactiferous sinus was not clinically benefited by Novoxil. Table 1 summarizes the results of this method of treatment. It should be noted that while only 11 of the 21 quarters were found free of the organism after freshening, the milk appeared normal and had a normal reaction in all but 4 quarters. Thus, if clinical manifestations or pH tests were used to ascertain the effect of treatment, the apparent results would be much better than if bacteriological culture methods were used. Chlorides and catalase content were within the normal limits in 6 uninfected and 2 infected quarters of 10 tested by those methods.

The 4 cows identified by S in table 1 were from a herd in which 9 infected cows were left untreated. Two of these made an apparent spontaneous recovery during a 256-day interval in which cultural tests were made. During the same interval, 3 of the 4 treated cows were found negative for streptococci in the lactation following treatment. Tests made on herd M, in which 2 cows were treated, were not sufficient to allow a comparison of results with the untreated group.

DISCUSSION

These few injection trials seem to indicate to the authors that at least part of the bactericidal action of Novoxil on lactating udders may be due to inflammation produced in the secreting and duct tissues. However, the establishment of this as fact

TABLE I.—A summary of the quarters treated while dry, the number of days from the time of treatment to the time of freshening, and the results of the injections.

Cow	Quarters Injected	Days Before Fresh	Reaction to Injection	Tests on Milk After Calving				
				Physical Appearance	pH	Cl %	Catalase % O_2	Cultural for S. agalactiae*
S53	RF	53	Slight swelling	Normal	Normal	0.103–0.107	3–12	Negative
	RR	53	Slight swelling	Normal	Normal	0.114–0.149	23–33	Negative
S67	RF	63	Slight swelling	Abnormal	Abnormal	0.099–0.206	50–210	Positive
	RR	63	Slight swelling	Normal	Normal	0.096–0.156	30–120	Positive
	LR	63	Slight swelling	Abnormal	Abnormal	0.103–0.241	26–200	Positive
	LF	63	Slight swelling	Normal	Normal	0.103–0.149	75–80	Positive
SW**	RF	56	Slight swelling	Normal	Normal	0.248	150	Negative
	RR	56	Slight swelling	Normal	Normal	0.185	30	Negative
	LR	56	Slight swelling	Normal	Normal	0.163	100	Negative
	LF	56	Slight swelling	Normal	Normal	0.185	120	Negative
S1	RF	81	Slight swelling	Normal	Normal	Not taken	Not taken	Negative
	LR	81	Slight swelling	Normal	Normal	Not taken	Not taken	Negative
	LF	81	Slight swelling	Normal	Normal	Not taken	Not taken	Negative
M2	RF	35	Moderate swelling	Normal	Normal	Not taken	Not taken	Positive
	RR	35	Moderate swelling	Normal	Normal	Not taken	Not taken	Positive
	LR	35	Moderate swelling	Abnormal	Abnormal	Not taken	Not taken	Positive
	LF	35	Moderate swelling	No secretion				
MF2	RF	55	Moderate swelling	Normal	Normal	Not taken	Not taken	Positive
	RR	55	Moderate swelling	Normal	Normal	Not taken	Not taken	Positive
	LR	55	Moderate swelling	Normal	Normal	Not taken	Not taken	Negative
	LF	55	Moderate swelling	Normal	Normal	Not taken	Not taken	Negative

Where 2 values of chlorides and catalase are given, the former refers to the first test after calving and the second to a later test. The first 4 cows were in herd S and the last 2 in herd M.

RR = right hind; RF = right fore; LR = left hind; LF = left fore.

*Tests used were the Hotis test and a microscopic examination of stained smears from the Hotis tube, supplemented in a few samples with streaking on the blood agar plate.

**The cow SW was tested for chlorides and catalase seven days after parturition while her udder still showed edema, and the values reflect what probably was a physiological departure from the normal.

would not necessarily contraindicate the use of Novoxil suspension, providing the infection was eliminated in a fair proportion of cases. Treatment of dry quarters does not seem to result in clinical inflammation, but a definite bactericidal action was evident.

In a comparison of the two different times of treatment, injection during lactation has the following disadvantages:

1) Greater expense because more Novoxil is used.
2) Inconvenience of several trips if only one cow is treated.
3) Greater liability of introducing a new infection on the cannula.
4) Loss of the whole production of the cow for about two weeks and some loss thereafter.
5) A lower percentage of cures, as reported by Schalm.[11]

[11]Schalm, O. W.: The Treatment of Chronic Bovine Mastitis. J.A.V.M.A., *100* (1942), pp. 323-334.

The advantages of treatment during the lactation period over the dry period are:

1) All the infected cows in a herd can be treated at once.
2) Injections can follow reliable diagnostic tests by only a few days.
3) Quarters may be saved that would be lost if allowed to continue with recurrent inflammation for several months of a lactation.

SUMMARY

Novoxil treatment of 3 quarters of 2 cows infected with *Streptococcus agalactiae* produced an acute clinical inflammation with secretions containing abnormally large amounts of catalase and chlorides. The number of streptococci eliminated was reduced markedly, but a few were found in the secretion of all 3 quarters sixteen days later, just before the cows were slaughtered. Postmortem gross and histopathologic examinations disclosed acute inflammatory changes which were more severe in the

Sulfaguanidine as a Prophylactic During the Period of Acquirement of Resistance by Chickens to Cecal Coccidiosis

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JOHNSON,[1] Tyzzer,[2] and Tyzzer, Theiler, and Jones[3] demonstrated experimentally that chickens developed a high degree of resistance to cecal coccidiosis as a result of the administration to them of numbers of sporulated oöcysts of *Eimeria tenella.* Following this discovery, these investigators suggested the possibility of immunizing chickens early in life as a means of reducing the mortality from this disease on the range. No doubt, this suggestion supplied the stimulus for considerable investigational work, notably that of Jankiewicz and Scofield[4] on the administration of heated oöcysts of *E. tenella,* and of Waxler[5] and Waxler and Herrick[6] on the inoculation of oöcysts attenuated by x-rays. It is at once obvious that were either of the above methods adopted for the establishment in chickens of a resistance to coccidiosis, the cost of the labor involved in treating birds individually would be prohibitive.

Investigations have shown that in order to become resistant to cecal coccidiosis, chickens must develop at least a mild form of the disease. Since coccidial infections, as they occur naturally, are not limited to the mild form, the mortality is usually high. It follows, therefore, that some means of protection against fatal doses of coccidia must be afforded during the period of immunization.

Recent work by Levine[7] and by Farr and Allen[8] demonstrated that sulfaguanidine was effective in preventing cecal coccidiosis and indicated that the degree of protection bore a direct relation to the amount of drug administered. Farr and Allen reported that birds which had been protected to the extent of showing no clinical symptoms, by the administration of either a 1 or a 2 per cent concentration of sulfaguanidine, were highly susceptible to reinfection one month after cessation of treatment. This degree of susceptibility was attributed to the practically complete protection from infection which the high concentrations afforded. The possibility that a lower concentration of sulfaguanidine might hold the disease in check and, at the same time, permit infections to develop, seemed worthy of investigation.

The experiments described in this paper were undertaken to determine, therefore, the value of a 0.5 per cent concentration of sulfaguanidine as an aid in the establishment of resistance to coccidiosis in young chickens which were confined in pens in which oöcyst-contaminated litter was present.

EXPERIMENTAL METHODS

The diet of the birds, method of obtaining and counting oöcysts, and the method of inoculation have been described in a previous paper.[8] The birds used in these experiments were obtained as 1-day-old chicks from the

The experiments described in this paper were conducted at the United States Department of Agriculture, Beltsville Research Center.

From the Zoölogical Division, United States Bureau of Animal Industry, Beltsville Research Center.

(Continued from preceding page)

2 quarters clinically abnormal before treatment than in the quarter which was giving normal milk prior to the injections. Injection of 21 dry quarters of 6 infected cows resulted in the elimination of the infection in 11 quarters, with very little or no inflammation produced.

ACKNOWLEDGMENT

The writers are indebted to Dr. B. B. Morgan, Professor E. G. Hastings, and Professor I. L. Baldwin for suggestions, and to Dr. Morgan for aid in preparing the graph.

Animal Husbandry Division of the Bureau of Animal Industry. They were placed in an isolation room in clean all-metal brooders. During the period preceding each experiment, repeated fecal examinations made by the sugar flotation method failed to reveal the presence of oöcysts.

The medicated mash was prepared by adding 0.5 per cent by weight of sulfaguanidine to the regular mash and mixing thoroughly.

The contaminated litter was prepared by scattering clean dry litter evenly over the floors of two rooms of equal size and allowing an equal number of birds previously inoculated with the same number of oöcysts of *E. tenella* to remain on the litter for at least two days after oöcysts began to appear in the feces. Both groups were removed the day before the experimental birds were placed in the contaminated pens.

In each of 2 experiments, 11-day-old Rhode Island Red chickens were divided into 3 groups of equal size and approximately equal weight. The members of group 1 were given medicated mash for three days before and fourteen days after being placed on the oöcyst-contaminated litter. The members of group 2 were handled in a manner identical with that of group 1, except that no drug was added to their mash. The members of group 3 were not exposed to infection until the thirty-eighth day of the test. During these experiments, all birds were weighed on the first and fourth days and every seventh day thereafter. The weight data were analyzed* by the analysis of variance method as described by Snedecor.[9]

Twenty-one days after the removal of the medicated mash from the members of group 1 (thirty-eighth day of experiment), the birds of all 3 groups were inoculated with oöcysts to determine whether they were resistant to cecal coccidiosis. The surviving birds of each experiment were necropsied on the forty-fifth and forty-sixth days, and a comparative study was made of the macroscopic lesions in the various groups. All birds were examined for gross lesions of intestinal coccidiosis and, in addition, scrapings from the small intestine of representative birds from each group were examined by sugar flotation for the presence of oöcysts of other species of coccidia.

EXPERIMENTAL DATA

Experiment 1.—In this experiment, each of the 3 groups consisted of 49 birds until the end of the third week, when 1 of the birds of group 3 became crippled and was eliminated. At the time they were placed on the litter, birds of groups 1 and 2 were given an initial inoculation of 7,700 oöcysts as a means of increasing the severity of the disease. A litter of wood shavings was used and, in order to minimize the loss of the vitality of the oöcysts due to desiccation, water was added occasionally from a sprinkler. The birds of group 3 were confined in wire cages in a separate room. A single dose of 119,000 oöcysts was administered to each bird of the 3 groups to test its resistance to infection.

Following exposure to infection, 28 of the 49 birds fed the regular mash (group 2) died. The deaths occurred on the fifth, sixth, and seventh days after the initial exposure. In the treated group (group 1), 5 of the 49 birds died, the deaths occurring on the fifth and sixth days after the initial exposure. Following the inoculation made on the thirty-eighth day of the experiment, 12 of the 48 birds, which had been kept free of coccidia (group 3) until the time of this inoculation, died. All survivors of this group were found to have extensive lesions characteristic of cecal coccidiosis when necropsied on the eighth day after inoculation. In contrast to the condition found in group 3, the birds of groups 1 and 2 had only minor lesions at the time of necropsy. The cecums of 82 per cent of the birds in group 1 and 57.1 per cent of those in group 2 appeared to be normal.

As shown in table 1, weight data were recorded only for groups 1 and 2. Those of group 3 were not considered because the conditions under which the birds had been confined were not comparable to those of groups 1 and 2. By the end of the thirty-eighth day of the experiment, the birds of group 1 had gained an average of 24.7 Gm. less than those of group 2, a difference not shown to be statistically significant. During the week following the final inoculation on the thirty-eighth day, the members of group 1 had gained an average of 16.8 Gm. less than those of group 2. However, group 1 gained an average of 13.2 Gm. less than group 2 during the week preceding inoculation. The differences in weight gains

*The writers wish to acknowledge the assistance of Mr. A. G. Dinaburg of the Zoölogical Division in analyzing the data.

[9]Snedecor, G. W.: Statistical Methods Applied to Experiments in Agriculture and Biology. Iowa State College Press, Ames, Iowa (1940).

TABLE I.—Results of Feeding Sulfaguanidine to Chickens Confined on Eimeria Tenella Contaminated Litter and the Effects of the Drug on the Acquisition of Resistance.

Experiment No.	Group No.	Amount of drug added to mash	No. of birds Start of experiment[1]	No. of birds End of experiment	Mortality after first exposure	Mean gains in Gm. of survivors First 38 Days	Mean gains in Gm. of survivors Next 7 days	Mean gains in Gm. of survivors Entire period	Reinoculation (after 38 days) Mortality	Reinoculation (after 38 days) Lesions
	1	0.5 %, 3 days before and 14 days after			%				%	
1	2	exposure	49	44	10.2	504.8	146.4	651.2	None	Slight
		None	49	21	57.1	529.5	163.2	692.7	None	Slight
	3	None	48	36	Not exposed	....	....	....	25.0	Extensive
	1	0.5 %, 3 days before and 14 days after			None	503.4	137.1	640.5	None	Slight
2	2	exposure	40	40						
		None	40	31	22.5	509.1	133.5	642.6	None	Slight
	3	None	40	35	Not exposed	525.9	−24.6	501.3	12.5	Extensive

[1]Eleven days old.

during the week before and the week after inoculation were found to be significant. It is believed, therefore, that the difference in weight gain between the 2 groups during the final week was probably a continuation of a growth differential which existed before the inoculation and was probably not the result of a retarding effect on growth due to the direct effect of coccidial lesions. This view is supported in part by the evidence cited above, namely, that the members of group 1 had less extensive coccidial lesions at necropsy than those of group 2. It is possible that the growth differences between the 2 groups, both before and after the final inoculation, were due to the fact that the 44 surviving birds of group 1 and the 21 survivors of group 2 were confined to pens of equal area. Thus, the latter group had the advantage in that more space was available to each bird.

Experiment 2.—Each of the 3 groups in this experiment consisted of 40 birds. Those of groups 1 and 2 were not inoculated at the time they were placed on contaminated straw litter. No water was added to the litter at any time during this experiment. The birds of group 3 were confined on clean straw litter in a room comparable in size to those in which groups 1 and 2 were kept. Each bird of the 3 groups received a single dose of 133,000 oöcysts in order to test its resistance to infection.

In this experiment, 9 of the 40 birds fed the regular mash (group 2) died of cecal coccidiosis after they had been placed on the contaminated litter. The deaths occurred on the sixth, seventh, eighth, and tenth days after exposure. There was no mortality in the treated group (group 1) during the entire period of the experiment. After the test inoculation on the thirty-eighth day, 5 deaths occurred in the group which had been kept free from *E. tenella* infection (group 3); all the survivors in this group were severely affected and at necropsy were found to have extensive lesions of cecal coccidiosis. As in experiment 1, the members of groups 1 and 2 were only slightly affected. No deaths occurred among these birds, and at necropsy it was found that the cecums of 72.5 per cent of the birds in group 1 and 51.6 per cent of those in group 2 were normal in appearance.

By the end of the thirty-eighth day of the test, the birds of group 1 had gained an average of 5.7 Gm. less than those of group 2. During the week following the test inoculation, the birds of group 1 gained an average of 3.6 Gm. more than those of group 2. During the final week, birds of group 3 lost an average of 24.6 Gm. In this experiment, where the numbers of surviving birds in groups 1 and 2 were more comparable than in experiment 1, the differences in mean gains during the

first thirty-eight days and during the final week were small and not significant. This fact is of interest in the light of previous work,[8] which indicated that a retardation in growth rates is caused by the administration of sulfaguanidine at the 1 and 2 per cent levels. It is interesting to note that at the end of the thirty-eighth day, the mean gains of the birds in group 3 did not differ significantly from those of the surviving birds of groups 1 and 2, presumably because the members of the latter 2 groups had sufficient time to regain weight lost during the course of the disease.

Shortly before the final inoculation, routine examination of the droppings of birds in group 3 revealed the presence of a few oöcysts which were identified as belonging to species other than *E. tenella*. However, since gross lesions caused by species other than *E. tenella* were not found in birds of either of these experiments, it is considered unlikely that the results were significantly affected.

DISCUSSION AND CONCLUSIONS

The greater severity of infections in the treated and untreated groups (groups 1 and 2) of experiment 1 may be attributable to the additional oöcysts administered to the birds at the time they were placed on the infective litter. In neither of experiments 1 and 2 did deaths occur after ten days from the time of the initial exposure to oöcysts, thus indicating that a resistance was acquired promptly in both groups. No deaths occurred in group 1 of experiment 1 after the sixth day, indicating that the acquisition of resistance in this group was just as prompt as in group 2 where the last death occurred on the seventh day. Furthermore, no deaths occurred in group 1 after the birds were taken off the medicated mash.

The fact that more birds in group 1 of each experiment were free from gross coccidial lesions at the time of the final necropsy than were those of group 2 cannot be explained on the basis of the evidence at hand.

The results of these experiments indicate that the use of a 0.5 per cent concentration of sulfaguanidine holds promise as an aid in establishing resistance to cecal coccidiosis.

SUMMARY

1. Sulfaguanidine, administered in mash at a concentration of 0.5 per cent for three days before and fourteen days after young chickens were exposed to infection by means of inoculation and exposure to contaminated litter or by exposure to contaminated litter alone, reduced markedly the mortality from cecal coccidiosis.

2. Chickens so treated with sulfaguanidine and held on infective litter for twenty-one days after the cessation of treatment were fully as resistant to subsequent infection as the surviving untreated controls.

3. The weight gains of chickens so treated and held on infective litter for twenty-one days after the cessation of treatment were not significantly different from those of surviving untreated controls.

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[5]Waxler, S. H.: Immunization against Cecal Coccidiosis in Chickens by the use of X-ray Attenuated Oöcysts. J.A.V.M.A., *99* (1941), pp. 481-485.

[6]Waxler, S. H., and Herrick, C. A.: Immunization against Coccidiosis by the use of X-ray Attenuated Oöcysts. J. Parasitol., *27* (Dec. 1941), Suppl. 17.

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Identification of Eggs of Nematodes Parasitic in Domestic Sheep

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IT IS WELL known that parasitism due to nematodes or roundworms is one of the primary causes of losses in sheep in the United States and other sheep-raising countries. One of the important and useful techniques in the diagnosis of worm parasitism in sheep, from the qualitative as well as quantitative standpoints, is the examination of fecal material for eggs. It is evident that if accurate diagnosis of nematode infections can be made by fecal examination and clinical manifestations, the need for sacrificing animals from a flock to determine whether unthriftiness and death losses are due to parasites can be partially or wholly eliminated.

A method of diagnosing nematode parasitism in sheep by fecal cultures and the examination of the infective larvae was described by Dikmans and Andrews[1] and Andrews.[2] This method is useful in verifying egg determinations, but employed alone is too time-consuming and not well adapted to quick diagnosis of mixed infections. A detailed study was made by Shorb,[3, 4] on the eggs of various genera and species of nematodes of domestic ruminants, who found that reasonably accurate differential diagnoses and egg counts could be made. This was particularly true of nematodes found in sheep. For some time, it has appeared that the process of acquiring skill in the identification of eggs of various species of nematodes could be facilitated, and the time factor reduced, if photographs accompanied by brief descriptions of eggs of individual species were available.

Photomicrographs of the eggs of 14 species of sheep nematodes in stages typical of their appearance in fresh fecal material have been obtained, and these are presented together with descriptions and data of variations from the typical forms that have been observed by the writers over a period of several years. It is believed that with the assistance of descriptions, measurements, photomicrographs, and some practice, the various eggs of nematodes of sheep can be identified with reasonable accuracy in fecal preparations.

The species of nematodes discussed in this paper, together with their location in the alimentary tract, are as follows:

Species of Nematoda	Location in Host
Haemonchus contortus	Fourth stomach
Trichostrongylus axei	"
Ostertagia circumcincta	"
Ostertagia marshalli	"
Trichostrongylus colubriformis	Small intestine
Trichostrongylus vitrinus	"
Bunostomum trigonocephalum	"
Nematodirus spathiger	"
Nematodirus filicollis	"
Cooperia curticei	"
Strongyloides papillosus	"
Capillaria brevipes	"
Chabertia ovina	Large intestine
Oesophagostomum columbianum	"
Trichuris ovis	

MATERIALS AND METHODS

With the exception of one species (*Ostertagia marshalli*), the eggs of nematodes to be photographed were obtained by dissection of living females. As females of *Nematodirus filicollis* and *Nematodirus spathiger* cannot be readily ated except on the basis of egg size, as noted by Shorb[4] and by Tetley,[5] this character, in addi-

From the Zoölogical Division, Bureau of Animal Industry, United States Department of Agriculture.

[1]Dikmans, G., and Andrews J. S.: A Comparative Morphological Study of the Infective Larvae of the Common Nematodes Parasitic in the Alimentary Tract of Sheep. Amer. Micros. Soc. Trans., lii (1933), pp. 1-25.

[2]Andrews, J. S.: A Note on the Morphology of the Anterior Ends of the Infective Larvae of Some Nematodes Parasitic in the Alimentary Tract of Sheep. Helminthol. Soc. Wash. Proc., ii (1935), pp. 88-90.

[3]Shorb, D. A.: Differentiation of Eggs of Various Genera of Nematodes Parasitic in Domestic Ruminants in the United States. U. S. Dept. Agric., Tech. Bul. 694 (1939), 11 pp.

[4]*Ibid.*: A Comparative study of the Eggs of Various Species of Nematodes Parasitic in Domestic Ruminants. J. Parasitol., xxvi (1940), pp. 223-231.

[5]Tetley, J. H.: The Differentiation of the Eggs of the Trichostrongylid Species *Nematodirus filicollis* and *N. spathiger*. J. Parasitol., xxvii (1941), pp. 473-480.

tion to identification of males, was relied upon as a criterion of separation. Several sheep examined *post mortem* contained only *N. spathiger* males, and one examined was found to have only *N. filicollis* males. In these cases, it is likely that only females of the same species were present. The females of *Ostertagia circumcincta* and *Ostertagia trifurcata* cannot be differentiated by an means known at present. No infested sheep were found with only *O. trifurcata* males. The eggs of *O. circumcincta* photographed were dissected from females taken from sheep harboring only males of that species. No difficulties in the identification of other species were encountered. All identifications follow Ransom[6] except where generally approved taxonomic changes have been made by later workers.

Female worms were removed from the alimentary tract of freshly killed lambs or sheep. These were placed in physiologic saline and identified. Once the female worms were identified, one or more, as necessary, were transferred to a small quantity of physiologic saline on a glass microscopic slide and the solution ringed with vaseline. Here the eggs were carefully removed from the uteri with the aid of a dissecting microscope. The débris from the dissected worms was removed from the slide and eggs appearing most similar to those normally found in fresh feces were centered on the slide and sealed with a coverslip. To prevent further development, until photographed, this preparation was kept in a refrigerator at 33 F. The thin-shelled eggs may be preserved in good condition by this procedure for two weeks or more, and the thick-shelled eggs may be kept for photographing for several months. There are several advantages to this method; namely, (*a*) slides can be prepared over a period of several days and photographed on a later date; (*b*) a débris-free slide can be prepared to give a clear picture; (*c*) the eggs can be selected to show their typical appearance; and (*d*) there can be no question as to the origin of the eggs of a particular species.

As no living specimens of *O. marshalli* could be obtained, the photomicrograph of the egg of this species was made from preserved material prepared in the manner described above. It has so far been impossible to obtain photographs of the eggs of the common trichostrongyle, *Trichostrongylus vitrinus*. The eggs of this species, however, are similar to those of *Trichostrongylus colubriformis*, and a description is included in the text.

A photomicrographic camera attachment with an 8X ocular was used with a microscope provided with achromatic objectives. A small microscope lamp with a 100 watt Mazda projection bulb and a white ground-glass filter was used as a source of light. Medium commercial orthochromatic dry plates, 3¼"x4¼", were found to be very satisfactory.

In addition to photomicrographs of eggs of 14 nematode species, several photomicrographs of direct centrifugal flotation preparations of fecal material from heavily infested sheep are presented. Photographs of 9 of the 14 species as they appear in normal sugar flotations of fecal samples were made (fig. A, B, C, D, E).

When seen with the aid of a microscope and transmitted light, the eggs, or more properly the developing embryos within the eggs, of sheep nematodes in feces generally have a yellowish-brown color. The shade or depth of the coloration varies considerably with different species. Every effort was made in preparing the prints to represent correctly this difference in the depth of color. Of course, the brown coloration of the eggs appears black or gray, as the case may be, in the photomicrographs. It should be noted here that the photographs of the eggs of the 14 individual species were made with the optical combination of an 8X ocular and a 4 mm. objective. The resultant relatively high magnification tends to give an optical section of the eggs, while this condition is not so pronounced at lower magnifications. An operator using lower magnifications must, therefore, make some adjustment. Most of the eggs can be identified at a lower magnification than that represented in these photographs, but when any question as to the identity of a particular egg arises, a shift to a higher magnification can be made.

DESCRIPTION OF THE EGGS

Results of a detailed study of the eggs of various nematodes of domestic ruminants have already been published (Shorb[3, 4]). Consequently, the present paper will be confined to brief descriptions of the characteristics which serve to differentiate the eggs of nematodes most commonly encountered in sheep.

N. spathiger and *N. filicollis*.—Eggs of these two species are easily identified on the basis of differences in size and shape of shell (fig. 1, 2, E). The eggs of *N. spathiger* are larger (181 to 230μ long by 91 to 107μ wide) than those of *N. filicollis* (149 to 194μ long by 74 to 107μ wide). The eggs of *N. filicollis* have a uniformly smooth outline, while those of *N. spathiger* normally have tapering, more

[6]Ransom, B. H.: The Nematodes Parasitic in the Alimentary Tract of Cattle, Sheep, and Other Ruminants. U. S. Dept. Agric., Bureau Animal Indus. Bul. 127, (1911).

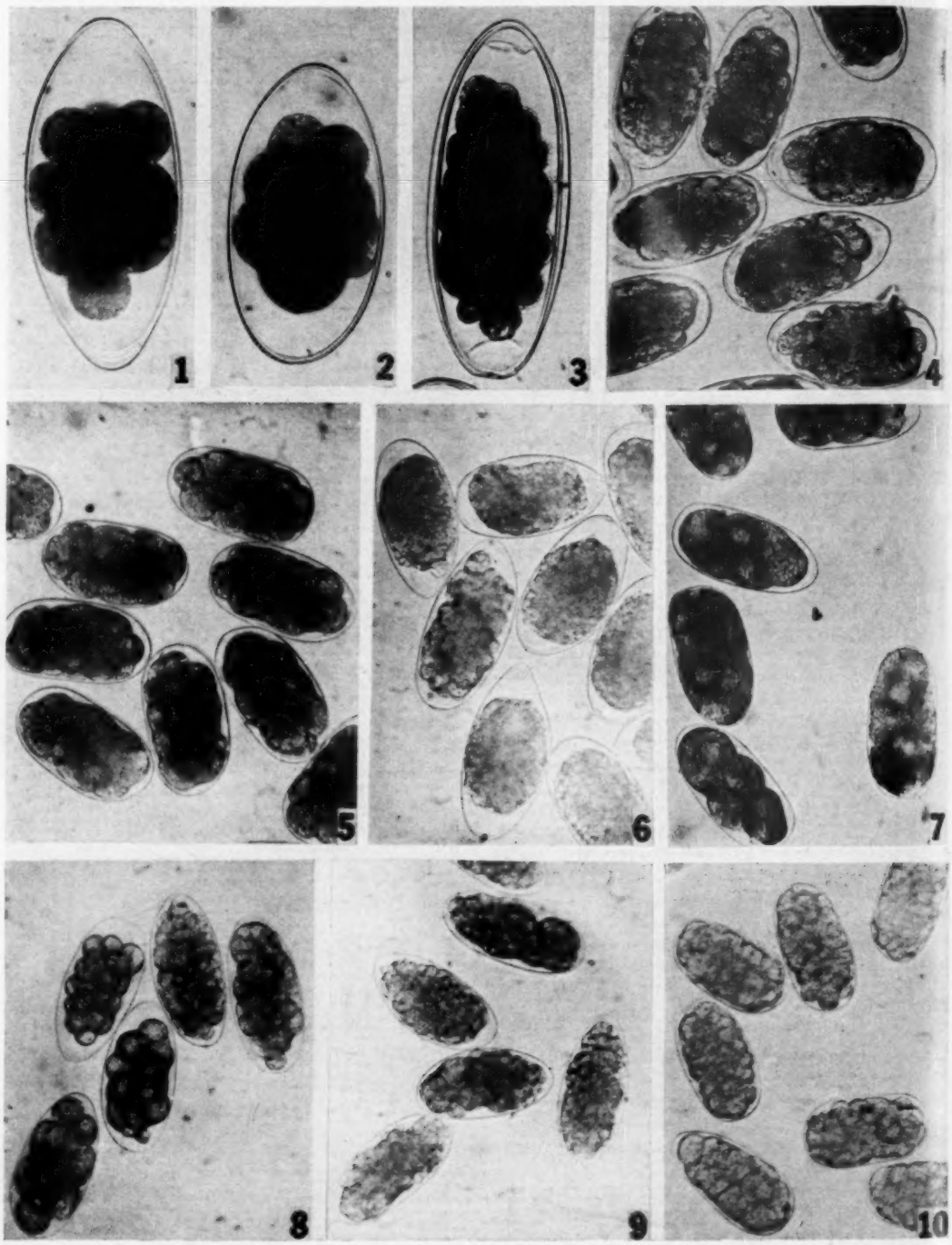

Fig. 1 to 14 on plates I and II are photomicrographs of eggs of some 14 species of nematodes commonly found in sheep. All were photographed at the same magnification and were arranged on the plates in a series from the largest eggs to the smallest. Fig. 1.—Nematodirus spathiger; fig. 2.—Nematodirus filicollis; fig. 3.—Ostertagia marshalli; fig. 4.—Chabertia ovina; fig. 5.—Bunostomum trigonocephalum; fig. 6—Ostertagia circumcincta; fig. 7.—Oesophagostomum columbianum; fig. 8.—Trichostrongylus colubriformis; fig. 9.—Trichostrongylus axei; fig. 10.—Haemonchus contortus. Fig. 3 is photomicrograph of preserved material, others are photomicrographs of living eggs. Magnification of fig. 1 to 10 x244.

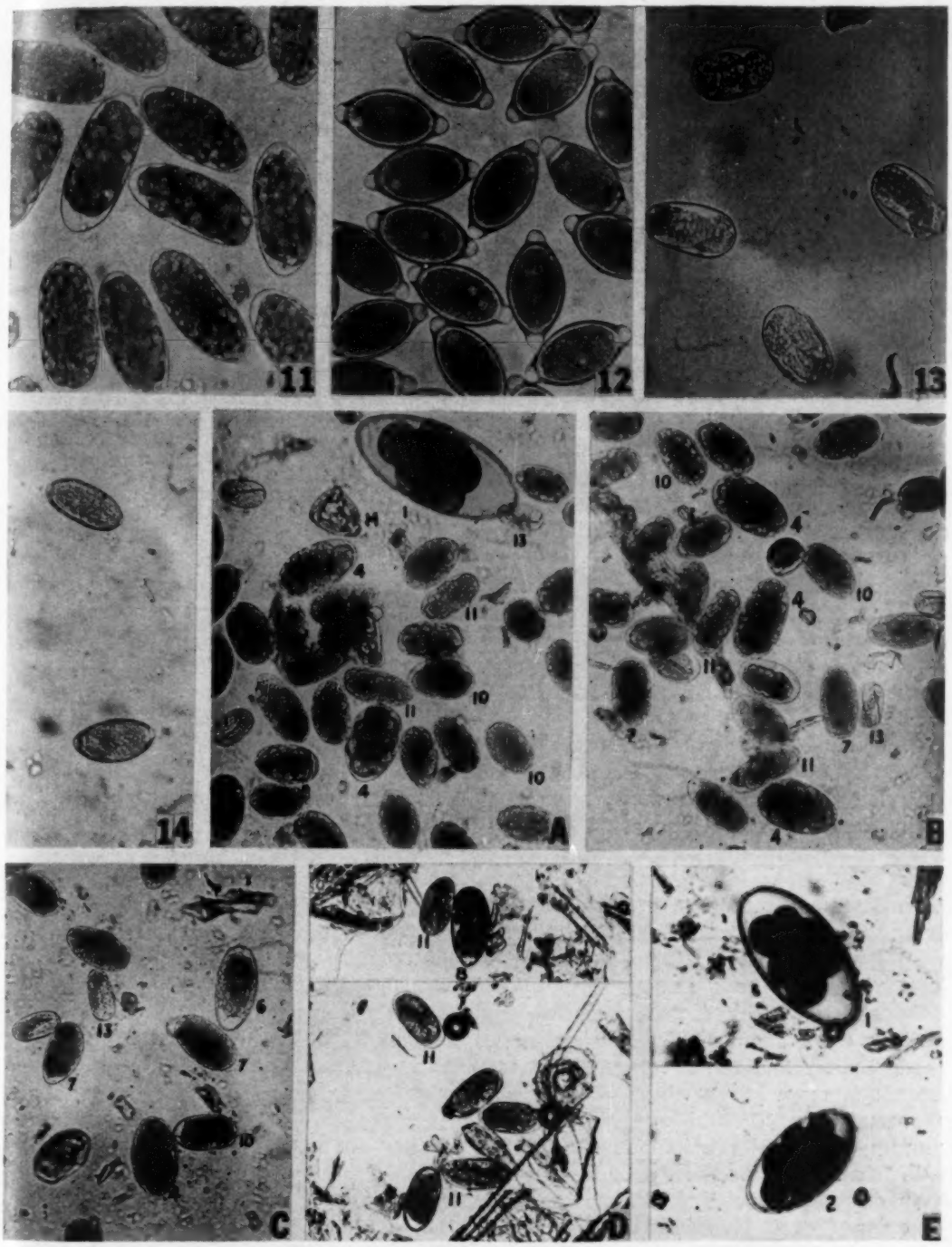

Fig. 11.—Cooperia curticei; fig. 12.—Trichuris ovis; fig. 13.—Strongyloides papillosus; fig. 14.—Capillaria brevipes. Magnification of fig. 11 to 14, x244. Fig. A to E are photomicrographs made at a lower magnification (x135) of direct centrifugal flotation preparations (using sugar solution, s.g. 1.4) of feces from sheep suffering from heavy mixed parasite infestations. In the case of fig. A, B, C, and E, fresh feces were used, while in the case of fig. D, feces kept 12 hours at room temperature were employed. Small numbers in fig. A to E correspond to the appropriate species in fig. 1 to 14. M in fig. A is an egg Moniezia expansa, sheep tapeworm.

or less thickened, ends. In fresh fecal material, the eggs of both species contain 1 to 8 dark-colored cells. A small percentage of the eggs of the two species may overlap in size; this overlapping was reported by Tetley[5] to be about 8.8 per cent. In rare cases, malformed eggs may be found, and Tetley[5] considers this an indication of an adverse host-parasite relationship.

O. marshalli and *O. circumcincta.*—*O. marshalli* produces very large eggs (fig. 3) (178 to 217μ long by 75 to 100μ wide), which have shells greatly thickened at the sides, but otherwise resembling those of Nematodirus. The eggs of *O. circumcincta* (fig. 6) are smaller (85 to 103μ long by 44 to 56μ wide) and have relatively thin shells. In the case of the former species, the morula is quite dark in color, while the morula of the latter eggs are further advanced in development and light in color; the light color of the eggs of *O. circumcincta* is important in differentiating them from those of other species having eggs of approximately the same size, such as *Bunostomum trigonocephalum* and *Chabertia ovina.*

C. ovina.—The eggs of *C. ovina* (fig. 4, A, B, C) are relative large (80 to 100μ long by 47 to 59μ wide) and most easily confused with those of *B. trigonocephalum.* However, with practice, the eggs of these two species can be differentiated on the basis of small differences. Those of *C. ovina* are usually oval shaped, while those of *B. trigonocephalum* have very blunt ends and straight sides; although eggs of both these species are dark in color, those of *C. ovina* appear a shade lighter and the development of the morula is more advanced. Relatively few abnormal eggs of *C. ovina* have been encountered in fecal examinations.

B. trigonocephalum.—Eggs of this species have dimensions quite similar to those of *C. ovina* (88 to 104μ long by 74 to 56μ wide). The embryo, however, consists of only 8 to 16 cells and is very dark in color, often appearing almost black (fig. 5). The ends of the egg shell are usually blunt and the sides parallel, in contrast to the usual oval shape of the eggs of *C. ovina.* Few abnormal eggs have been found in fecal examinations.

Oesophagostomum columbianum. — The eggs of this species are smaller (fig. 7, B, C) (74 to 88μ long by 45 to 54μ wide) and are most easily confused with those of *Haemonchus contortus.* However, their normal deep color, small number of cells (4 to 16) and poorly defined cell membranes are definite distinguishing characteristics. Variations in depth of color sometimes occur in eggs from different fecal samples, but when variations do occur, the normal coloration relative to other species is maintained. This condition must be guarded against in differentiating eggs that resemble each other closely and is especially important in the case of eggs of *O. columbianum* and *H. contortus.*

H. Contortus.—The eggs of *H. contortus* (fig. 10, A, B, C) are usually slightly smaller and appear to be more rounded than those of *O. columbianum.* They vary in length from 65 to 82μ and in width from 39 to 46μ. In fresh material they are found in the morula stage and are lighter in color than eggs of *B. trigonocephalum* and *O. columbianum* with which they may be confused. Some slight variations may occur in respect to shape and coloration of these eggs.

T. vitrinus, T. colubriformis, Trichostrongylus axei.—Trichostrongyle eggs (fig. 8, 9, D) are all characteristically irregular in shape, and the main variation among the eggs of these three species is size, those of *T. vitrinus* being 93 to 118μ long by 41 to 52μ wide, *T. colubriformis* 79 to 101μ long by 39 to 47μ wide, and *T. axei* 79 to 92μ long by 31 to 41μ wide. *T. vitrinus* produces the largest eggs of the three species. They are distinguished by their long narrow shape, and have one end pointed and the other rounded. The eggs of *T. colubriformis* (fig. 8) are slightly smaller than those of *T. vitrinus* and less pointed. The eggs of *T. axei* (fig. 9) are the smallest of the three species, and, except for their irregular shape and pointed end, closely resemble those of *Cooperia curticei.* All these eggs are in the morula stage at the time of passage from the host. The eggs of *T.*

vitrinus and *T. colubriformis* are normally slightly darker in color than those of *T. axei*. Large Trichostrongylus eggs may be confused with some irregularly shaped *O. circumcincta* eggs, but the latter invariably are lighter in color and more rounded. Trichostrongyle eggs have occasionally been observed in fecal preparations that were as much as twice as large as normal eggs; other distinguishing characteristics, however, are usually preserved.

C. curticei.—The eggs of this species are small (70 to 82μ long by 35 to 41μ wide) and closely resemble those of *H. contortus* in general appearance, but are more slender (fig. 11). The sides of the eggs are parallel and the ends usually blunt. In fresh material, they are in the morula stage, are light in color, and the morula closely resembles that of *T. axei*. In figure D are shown a number of *C. curticei* eggs that are partially embryonated; these were photographed after exposure of the feces to normal laboratory temperatures for several hours. It will be seen that they can still be differentiated from eggs of other species by their size and shape, as they are distinctly narrower than the eggs of *H. contortus*, Trichostrongylus spp., and *O. columbianum*.

Strongyloides papillosus.—The only nematode eggs of sheep that contain vermiform embryos when passed by the host (except those of Gongylonema spp., which are not considered here) are those of *S. papillosus*. The embryonated eggs are small (52 to 65μ long by 31 to 36μ wide) and easily differentiated from those of other species (fig. 13, A, B, C).

Trichuris ovis and *Capillaria brevipes*.—The operculated eggs of these two species are easily differentiated from each other and from those of other nematodes of sheep. The eggs of *T. ovis* (fig. 12) are larger (70 to 79μ long by 31 to 39μ wide) than those of *C. brevipes* (fig. 14) (49 to 53μ long by 24 to 26μ wide). The latter are the smallest eggs of any of the common sheep nematodes. The opercula of the eggs of *T. ovis* project from the surface of the shell, while those of *C. brevipes* are almost flush and have the appearance of a plug.

Recapitulation.—The eggs of *O. marshalli*, Nematodirus spp., Strongyloides, Capillaria, and Trichuris are relatively easy to differentiate from each other and from other helminth eggs commonly found in sheep feces. At first glance, the eggs of other species of roundworms commonly found in sheep appear very similar in size and shape, but after careful study, small differences in depth of color, shape, stage of development in fresh feces, and size become obvious. The eggs of these nematodes may be arranged in groups according to apparent depth of color as follows: Bunostomum, Chabertia, and Oesophagostomum eggs appear deep brown or nearly black; those of Haemonchus, Trichostrongylus, and Cooperia have a medium brown color; and those of *O. circumcincta* are light brown or almost colorless. As regards shape, the eggs of *O. circumcincta* and Chabertia are smoothly oval; those of Cooperia and Bunostomum are cylindrical with rounded ends; those of Trichostrongylus, asymmetrical and usually pointed; and those of Oesophagostomum and Haemonchus are rather square with rounded corners. The eggs of Haemonchus are usually more oval than those of Oesophagostomum. The square appearance of the eggs of Haemonchus and Oesophagostomum, however, is not always obvious. The stage of development of the embryos is also important as a differential character. The least developed embryos recovered from newly passed feces are found in eggs of Oesophagostomum (4 to 8 cells) and in eggs of Bunostomum (usually about 16 cells). The embryos in the eggs of Trichostrongylus, Chabertia, Haemonchus, Cooperia, and *O. circumcincta* are usually in the morula stage and so far developed that it is difficult to estimate the number of cells present. The size relationship between the eggs of these nematode species are illustrated in plates 1 and 2; the photomicrographs are arranged roughly according to size.

DISCUSSION

There are several points to consider and precautions to be taken in making accurate diagnoses of parasitism in sheep, based

upon differential identifications or counts of nematode eggs in fecal preparations. These are as follows: (1) Fresh fecal material or its equivalent (fresh feces kept in a refrigerator at 32 to 33 F. will remain in good condition for some time if sealed in a container to prevent evaporation) should always be used, since the difficulty in identification of the eggs increases as the development of the embryo progresses. (2) Counts of or examinations of slide preparations for eggs should be made immediately after preparation for the reason already given. When saturated NaCl solution is used, the protoplasmic material in eggs quickly plasmolyzes. When sugar solution is employed, plasmolysis is much less rapid. (3) A knowledge of the morphology of the adult worms is useful, as it is often desirable to check egg determinations against postmortem findings. (4) A thorough knowledge of the species of nematodes generally found in sheep in a particular region is also useful, as several species may be eliminated from consideration in this way. For example, eggs of *O. marshalli* and Nematodirus spp., being similar in size, may be confused when these species occur in the same animal. However, *O. marshalli* need not be considered in the eastern United States, as it is not found in sheep in this region. (5) Mistakes in identification can be corrected and confidence in one's ability to differentiate the eggs can be increased by occasional autopsy of animals shortly after egg determinations have been made.

SUMMARY

Photomicrographs of eggs of 14 common species of sheep nematodes and descriptions of eggs of 15 species are presented. Suggestions are also made which may aid in accurate identification of these eggs.

Establishment of Infection with Trichomonas Foetus in Bulls by Experimental Exposure

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THERE IS RELATIVELY little precise information concerning how infection with *Trichomonas foetus* becomes established in bulls. Presumably bulls become infected by coitus with infected heifers or cows. It appears to be well established, however, that once the infection is acquired by a bull, he usually carries it indefinitely. Because the bull is the usual source of infection for heifers and cows, it is necessary to determine the conditions under which bulls acquire the infection in order to provide a foundation for a logical approach to its prevention and control.

Rees and Garlick[1] reported the establishment of infection in a bull during the fall of 1936 or early part of 1937 through coitus with one or more infected females. Subsequently, Rees, Garlick and Avery (unpublished data in files of the Zoölogical Division) in work done at the Beltsville Research Center, failed to establish infection experimentally in any of 12 bulls exposed a total of 150 times to trichomonads by various methods, as follows: 69 exposures on all 12 bulls, made by introducing cultures of *T. foetus* into the prepuce; 40 exposures on 6 of the bulls by introducing into the prepuce fluid containing living Trichomonas from the vagina or uterus of infected females; 4 exposures on 2 of the bulls by introducing preputial washings from an infected bull into the prepuce; 23 exposures on 4 of the bulls by breeding to infected females having trichomonads in the vagina at time of service; and 14 exposures on 4 of the bulls by breeding to uninfected females immediately following their service by an infected bull.

The failure to infect these bulls indicated that they were refractory, at least under the conditions of these experiments. It is not clear from the evidence at hand whether this was due to natural resistance, or to insufficient opportunity for the trichomonads to become established. In order to determine which of these possibilities was correct, this work was continued in a second series of experiments. These experiments were designed to determine whether improved methods of experimental exposure would result in bulls becoming infected, and to obtain information on natural transmission of infection to bulls.

MATERIALS AND METHODS

The 6 bulls used in these experiments included 2 grade Jerseys, 2 grade Guernseys, 1 grade Holstein-Friesian, and 1 purebred Guernsey, the ages of these animals ranging from 14 months to 7 years. The purebred Guernsey had been retired from a herd because of a bad breeding record associated with trichomoniasis. Shortly after he was obtained for the experimental work on trichomoniasis, he recovered spontaneously from the infection. None of the remaining bulls had been infected previously, 4 had never served, and 1 had been bred 4 times to uninfected heifers.

In the natural transmission experiments, the bulls were bred to heifers known to have, or suspected of having, trichomonads in the vagina. One bull served 4 infected heifers in estrum, each of whose vaginal samples on the day she was served was positive. A second bull was force-bred to 3 infected heifers, and bred to 1 infected heifer probably in estrum; each of these heifers had large numbers of trichomonads in the vaginal sample on the day she was bred. A third bull was bred to 3 uninfected heifers immediately after services by an infected bull. Two of these 3 bulls were also later used in the second group of experiments.

A second series of exposures of bulls was made by introducing material containing tricho-

This work was carried out at the United States Department of Agriculture Beltsville Research Center, Beltsville, Md.

From Zoölogical Division, Bureau of Animal Industry, U. S. Department of Agriculture.

[1]Rees, Charles W., and Garlick, George C.: Experimental Transmission of Bovine Venereal Trichomoniasis. J. Agr. Research, lix (1939), pp. 769-775.

monads into the prepuce. The aim in these experiments was to locate large numbers of active trichomonads in the posterior portion of the prepuce long enough to allow them to become established. The trichomonads were obtained by flushing the vagina of a heifer or cow in an active stage of experimental infection with 7 cc. of sterile 0.7 per cent saline solution injected and retrieved by means of a vaginal pipette similar to the one described by Andrews and Miller (1938). The amount of fluid recovered from the vagina varied from 2.5 to 15 cc. The concentration of trichomonads in the sample was estimated by means of a hemacytometer. In most samples used for experimental infection of bulls, there were from 300,000 to 3,000,000 trichomonads per cc. of fluid. One or two pledgets of cotton were then soaked in the fluid containing trichomonads, and the pledgets inserted immediately with long-handled forceps into the prepuce of the bull to a position between the fornix and the tip of the glans penis in its retracted position. To avoid contamination of the moist pledgets with foreign material during insertion into the preputial orifice, a Graves vaginal speculum was used. After completion of the operation, the bull was placed in a stall overnight to lessen the chance of early expulsion of the pledgets. The pledgets were found to remain in the prepuce for at least one to two hours following the operation, while the bull was kept under close observation, and in some cases, the pledgets could still be palpated in the prepuce as long as twenty-four hours after they were inserted.

In a few cases, a slightly modified procedure was used. In 2 exposures of 1 bull, the penis was unsheathed by manipulation and rubbed with a towel prior to insertion of the pledgets. In 3 exposures of 3 different bulls, under epidural anesthesia, the penis was unsheathed to expose the entire preputial mucosa, and a thin layer of cotton soaked in vaginal washings was then wrapped around the glans before replacing the penis. Difficulty in maintaining the cotton at this location during and after retraction of the penis was encountered.

In the majority of cases, the introduction of infective material into the prepuce was repeated once or twice on successive or alternate days. In computing the total exposures for an animal, such a series is considered as a single exposure. If no infection became established in four to six weeks, the bull was given another exposure. The bulls suffered no apparent ill effects from the procedures used.

Weekly examinations for trichomonads were made of each bull during the entire period covered by the experiments, using a pipette especially designed for this purpose. This pipette is a modification of the vaginal pipette described by Andrews and Miller (1938). The preputial pipette used was longer than the vaginal pipette, had a smaller degree of bend, and the opening was at the end rather than on the side (fig. 1). It was used in the earlier part of the work by injecting saline solution into the prepuce and recovering as much of it as possible, much in the same manner as the vaginal pipette is used. Later, it was found that an equally good sample could be obtained by inserting the pipette into the prepuce without saline solution, aspirating exudate from the space around the glans and washing this out of the pipette into a test-tube with a few cc. of saline solution. Samples were examined within two hours and again after twenty-four hours, in saline-egg medium (Avery and Garlick[2]). The tubes were allowed to stand for about an hour to permit the trichomonads, if present, to become concentrated in the upper layer of the accumulation of preputial cells at the bottom of the tubes.

Fig. 1.—Pipette used in obtaining samples from the prepuce of bulls. Dimensions of pipette are: Length, 21 in.; diameter, 8 mm.

RESULTS

Four bulls did not develop infections, although they were repeatedly exposed to *T. foetus*, while the other 2 became infected following 1 exposure. The records for these 2 bulls follow.

The first bull, a 1½-year-old grade Guernsey, was first found to be infected a month after the first exposure; he apparently lost this infection following a second exposure, but became reinfected within a week following a third exposure (bull 207, table 1). In the first exposure, pledgets containing 2 cc. of vaginal washings from

[2]Avery, John L., and Garlick, George G.: A Simple Medium for the Cultural Diagnosis of *Trichomonas foetus* in Cattle. Proc. Helm. Soc. Wash., vii (1940), pp. 27-29.

TABLE I.—Results of Exposure of Bulls to Trichomonas Foetus.

Bull (No.)	Date of Birth	History Previous to Exposure	Exposures by Service to Infected Females	Exposures by Introduction of Trichomonads into Prepuce	Total Exposures	Results
			(Date)	(Date)		
194	8/17/39	Had never served	9/2/41[1] 9/6/41[1] 9/9/41[1]	10/20/41; 10/21 12/9/41 1/16/42; 1/17; 1/19 2/23/42; 2/24 3/27/42	8	Negative
210	7/24/40	Had never served	9/19/41 9/23/41 10/1/41	1/9/42; 1/10 2/10/42 4/17/42	6	Negative
207	5/19/40	Had never served		9/2/41; 9/5; 9/10 10/7/41 11/24/41; 11/25/41	3	Positive
209	10/29/34	Infection terminated spontaneously last positive examination 9/12/40		10/21/41; 10/22/41 12/30/41; 12/31; 1/1/42 2/12/42 4/7/42	4	Negative
214	12/3/40	Had never served	2/24/42 3/13/42 3/30/42 4/24/42		4	Negative
193	4/30/39	4 services to uninfected heifers		4/13/42	1	Positive

[1]Bred to uninfected female immediately following her service by an infected bull.

an infected cow were inserted into the prepuce, and this procedure was repeated twice within a period of eight days. Prior to the third insertion of pledgets, the penis was withdrawn by manipulation. Preputial washings obtained from the bull just before a second exposure, one month later, contained trichomonads, but unfortunately the exposure was made before the positive diagnosis of infection. In making the second exposure, the glans was withdrawn, the mucosa of the glans and prepuce irritated by rubbing and brushing, and preputial washings from an infected bull were injected into the prepuce by pipette. The following examinations were negative, and the bull did not transmit infection to a susceptible heifer to which he was bred a month after the exposure. It was concluded, therefore, that the infection which followed the first exposure had been lost. There was evidence of inflammation of the prepuce as a result of the procedure used in making the second exposure, which may have been a factor in the disappearance of the infection.

The third exposure of this bull, given seven weeks after the second, consisted of two introductions into the prepuce on successive days of pledgets soaked in 2 cc. and 3 cc. of vaginal washings containing 1,500,000 and 800,000 trichomonads per cc., respectively. The first examination, a week after the exposure, was positive for trichomonads, and the bull has been positive up to the present time (June, 1942), a total of about six months. There were no obvious clinical symptoms nor evidence of an acute phase of the infection. However, two weeks after the first positive examination, a hard tumorous area about a quarter of an inch in diameter appeared about an inch posterior to the preputial orifice in the tissue beneath the mucosa. This area increased to about a half inch in diameter before gradually disappearing about a month after it was first noticed. The superficial mucosa showed no erosion, and it is not certain whether there was any casual relationship between the presence of trichomonads and the tumorous area. Since becoming infected, the bull has been bred to 14 heifers and

cows. Three previously uninfected heifers all became infected following 1 service, and of the 11 previously infected heifers and cows, 6 became reinfected and 5 remained negative following 1 or more services.

The second bull, a 3-year-old grade Guernsey, developed infection within a week following the first exposure, and this infection is still present (June, 1942), two months after the exposure (bull 193, table 1). Infection was established following a single introduction into the prepuce of pledgets soaked in 5 cc. of vaginal washing containing 1,500,000 trichomonads per cc. from an infected heifer. A preputial sample taken with a dry pipette on the day following the exposure contained about 4,000 active trichomonads in 0.1 cc. of fluid, and all successive weekly examinations were positive. On the tenth day following his exposure, the bull was bred to a previously infected cow, and she became reinfected as a result of this service. No obvious clinical symptoms associated with the infection in this bull were observed.

None of the remaining 4 bulls became infected following 4 to 8 exposures each (bulls 194, 210, 209, and 214, table 1). Three of these bulls were exposed several times by the same procedure used in establishing infection in the 2 bulls already discussed, and once each by the method involving epidural anesthesia. In only 1 of the 3 bulls (bull 209) could the resistance to infection be associated with previous infection. Two of the bulls resistant to infection were about 14 months old at the beginning of experimental exposure, a third was about 2 years, and the fourth, previously infected, was about 7 years old. None of the exposures involving coitus with infected females, or with uninfected females following service by an infected bull produced infection.

DISCUSSION

The failure to produce any infections in the series of experiments involving 150 exposures on 12 bulls (Rees, Avery and Garlick, unpublished data) is indicative of the difficulty of experimentally infecting bulls with *T. foetus*. Negative results were also obtained by Walsh, McNutt and Murray[3] with a bull bred to infected females, and with a young bull injected in the prepuce with pus containing trichomonads; Staub[4] also failed to infect a bull by preputial injections of cultures.

That infection can be established experimentally in bulls was shown by Küst[5] who reported infecting a proved clean bull by injecting 25 cc. of pyometra material into the sheath. Abelein[6] reported that Thanner (unpublished dissertation) infected a bull by injection into the prepuce of a pure culture of trichomonads obtained from pyometra pus and cultured through 7 passages in horse-serum medium. Abelein also stated that Dangelmaier (unpublished dissertation) was able to establish infection in 3 of 9 bulls by the introduction of a small amount of a pure culture of trichomonads into the prepuce with a silk brush (Seidenpinsels). The establishment of infection in 2 of 6 bulls in the present work coincides with Dangelmaier's results so far as concerns the proportion of successful transmissions, although Dangelmaier used a different source of organisms and a different method of introducing them into the prepuce. It is possible that the failures to establish infections mentioned above were dependent in part upon inadequate methods of exposure.

Some investigators (Küst[5], Swangard[7]) reported symptoms of severe inflammation, including swelling of the prepuce, mucopurulent discharge from the prepuce, and disinclination to serve, following the establishment of infection in bulls. None of these symptoms was noted in either of the bulls which became infected during the experiments reported in this paper. Both bulls

[3]Walsh, F. E., McNutt, S. H., and Murray, Charles: *Trichomonas bovis* Infection in Cattle. Cornell Vet., xxiv (1934), pp. 60-74.

[4]Staub, Christian: *Trichomonas foetus* und ihre Beziehungen zum seuchenhaften Frühabortus und zur Sterilatät des Rindes. Inaug.-Diss., Zurich, (1936), 63 pp.

[5]Küst, Dietrich: Trichomonadenabort des Rindes. Deutsche tier. Wchnschr., xli (1933), pp. 785-789.

[6]Abelein, R.: Zur Krisis in der Beurteilung der Trichomonadenseuche des Rindes. Berl. u. Münch. tier. Wchnschr., xxii (May 30, 1941), pp. 261-265.

[7]Swangard, W. M.: Trichomoniasis in Cattle: Biological Studies and a System of Control. J.A.V.M.A., xcv (1939), pp. 146-151.

that became infected appeared normal in behavior and in appearance of the penis; however, the penis was not withdrawn under anesthesia in either bull, and it is possible that slight inflammation may have been present.

The failure of several of the bulls to become infected in spite of numerous exposures of the same kind that readily established infections in other bulls shows that there may be individual differences in resistance to infection. Age is probably not the determining factor, as indicated by lack of consistent differences in ages of the infected as compared with the resistant bulls, but it may be noted that the youngest bulls were in the noninfected group. It is probable that other factors, such as individual differences in anatomy and physiology of the prepuce, are of importance in determining these variations in resistance to infection.

Only negative information on conditions under which transmission to bulls occurs naturally was obtained in these experiments. The failure to establish infections in any of the exposures involving coitus shows that some bulls are resistant to infection by natural means. The results with experimental transmission indicate that individual variation in resistance of bulls is probably more important than factors involving the source of infection. This would explain why some bulls become infected by natural means while others do not.

SUMMARY

Five bulls were exposed to *Trichomonas foetus* by introducing into the prepuce cotton pledgets soaked in vaginal washings from experimentally infected heifers and cows. Two became infected; 3 remained negative after a number of exposures by this and other methods.

Thirteen bulls (including 12 from the unpublished work of Rees, Garlick and Avery) were exposed a total of 154 times by various methods not involving the use of pledgets. None became infected.

No obvious clinical symptoms associated with trichomoniasis were observed in the experimentally infected bulls. Each bull was able to transmit infection to susceptible cows by service.

The results indicate that there are distinct individual differences in natural resistance of bulls to infection with *T. foetus*.

Sulfaguanidine Therapy in Experimental Bovine Coccidiosis

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IN 1940, Marshall, Bratton, White, and Litchfield[1] reported that sulfaguanidine (sulfanilylguanidine) showed particular promise for the treatment of bacterial infections of the intestine. They pointed out two interesting properties of this drug, namely, (1) its low absorption from the alimentary tract and (2) its relatively high solubility in water. These properties suggested that sulfaguanidine might prove effective against intestinal coccidiosis, a disease against which flowers of sulfur, sulfanilamide, and sulfapyridine had already been reported by several workers[2] as being partially effective. Levine[3] and Farr and Allen[4] found sulfaguanidine to be an effective prophylactic against coccidiosis in chicks. The report of Foster, Christensen, and Habermann[5] that daily administration of the drug prevented lambs from acquiring natural coccidial infections suggested that sulfaguanidine might be successful against bovine coccidiosis.

The purpose of this paper is to report experiments in which treatment of experimentally induced bovine coccidiosis with sulfaguanidine gave promising results. A preliminary account of these experiments was published in abstract elsewhere.[6]

From the United States Regional Animal Disease Research Laboratory.

The writer is indebted to Mr. George D. Coffee for technical assistance.

[1]Marshall, E. K., Jr., Bratton, A. Calvin, White, J. H., and Litchfield, J. T., Jr.: Sulfanilylguanidine: A Chemotherapeutic Agent for Intestinal Infections. Bul. Johns Hopkins Hosp., *67* (1940), pp. 163-188.

[2]Levine, P. P.: Sulphur Therapy in Experimental Avian Coccidial Infection with *Eimeria necatrix*. Cornell Vet., *31* (1941), pp. 120-126.

[3]*Ibid.*: The Coccidiostatic Effect of Sulfaguanidine (Sulfanilylguanidine). *Ibid.*: *31* (1941), pp. 107-112.

[4]Farr, Marion M., and Allen, Rex W.: Sulfaguanidine Feeding as a Control Measure for Cecal Coccidosis in Chickens. Abstract in J. Parasitol., *27* (1941), p. 20.

[5]Foster, A. O., Christensen, John F., and Habermann, Robert T.: Treatment of Coccidial Infections of Lambs with Sulfaguanidine. Proc. Helminth. Soc. Wash., *8* (1941), pp. 33-38.

[6]Boughton, Donald C.: The Effects of Sulfaguanidine on Experimental Bovine Coccidiosis. Abstract J. Parasitol. *27* (1941), pp. 16-17.

Materials and Methods

The calves used in the present work were either Jerseys or Jersey-Guernsey crosses purchased when a few days old. Treated and control animals in each experiment were matched as to age, weight, sex, and uncontrolled exposure to coccidia prior to the beginning of the experiment. All calves received routine care as to housing and feeding. Daily records were kept, throughout the experiment, of the general appearance of the animal, consistency of the feces, and the types and number of oöcysts discharged. Differential oöcyst counts and estimates of the relative number of oöcysts were made by means of a modification of Christensen's[7] flotation technique. A standard volume of feces (1 level tablespoonful) rather than a standard amount of fecal sediment (1 cc.) was used in the present work. Oöcyst production was considered heavy when the estimated number of oöcysts in 1 drop (removed from the surface of the 15 cc. of flotation medium by means of a wireloop 0.5 cm. in diameter) exceeded 1,000. This figure, equivalent to a count of about 5,000 to 10,000 oöcysts per Gm. of feces, represents a level of oöcyst output which is often accompanied by clinical manifestations.

The inoculums were made up from fecal material collected from several infected calves. The material was stored in 2 per cent potassium dichromate at approximately 2 C. until a sufficient quantity was accumulated for experimental use. Ten to 15 liters of chromate-fecal suspension were usually required to provide the large numbers of oöcysts desired. The entire suspension used in making up the inoculums for a given experiment was placed in a flat-sided, 5-gal. oil can. The can was then tilted, by placing it in a rack constructed for the purpose, in such a manner that it rested on one bottom corner. A piece of glass tubing was inserted diagonally into the can through the opening in the top corner, the lower end of the tubing resting in the bottom corner of the can. A stream of air under pressure, introduced through the glass tubing, served to aërate and agitate the suspension. Sporulation of the oöcysts and thorough mixing of the material were accomplished simultaneously, a period of seven to ten days being sufficient to

[7]Christensen, John F.: The Effect of Copper Sulfate and Ferric Sulfate on Coccidial Oöcyst Output in Feeder Lambs. J.A.V.M.A., *96* (1940), pp. 478-480.

complete both processes. When maximum sporulation had been obtained, the contents of the can were divided into equivalent portions, which were stored in the refrigerator in individual containers until needed. Just before inoculation of the calves, the supernatant potassium dichromate solution was poured off; the fecal sediment, containing the oöcysts, was then washed and resuspended in warm water or milk. Each calf in a given experiment received one of the equivalent portions of the original suspension. Direct comparisons between resulting infections within the same experiment are therefore justified. Because the volume was too large for a single feeding, each portion was divided into several feedings, which were given over a two- or three-day period. The second day of such a period is considered as the first day after inoculation.

The sulfaguanidine was administered by capsule or was suspended in water and fed by nipple.

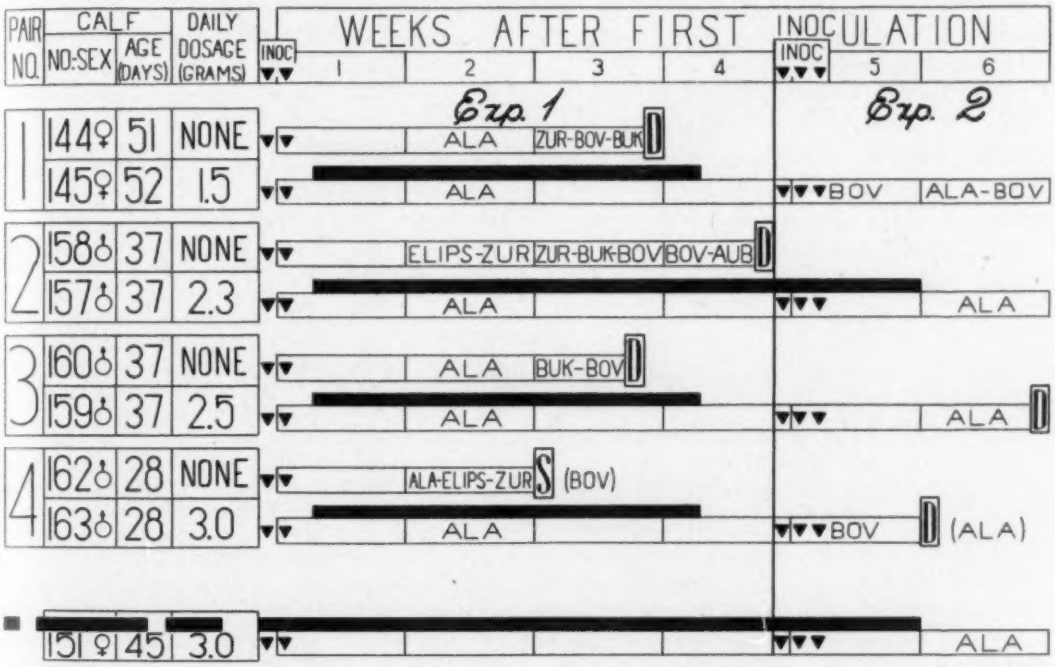
Experiment 1.—This experiment was designed to test the efficacy of sulfaguanidine against experimental mixed infections. The drug was administered in daily doses throughout the incubation period of the disease.

On July 12 and 13, 1941, 4 pairs of calves, ranging in age from 28 to 52 days, were inoculated with equivalent portions of a suspension of mixed species of bovine coccidia. *Eimeria alabamensis*, *Eimeria cylindrica*, *Eimeria bovis* (syn., *Eimeria smithi*), *Eimeria bukidnonensis*, *Eimeria auburnensis*, and *Eimeria zurnii* were all present in the inoculum. Each calf received approximately 50 million sporulated oöcysts. An unpaired calf, discussed in experiment 2, was likewise inoculated at this time.

On the second day after the inoculation had been completed, sulfaguanidine treatment was begun on the odd-numbered calves of the 4 pairs. The daily dosage was at the rate of 0.1 Gm. per 2.2 lb. of body weight. The drug was administered by capsule approximately thirty minutes before the morning feeding. All 4 treated calves received 21 consecutive daily doses. This constituted the extent of treatment for 3 of the calves; treatment was continued for twelve additional days on 1 calf for purposes of a second experiment, discussed elsewhere in this paper. The even-numbered calf of each pair received no drug.

The organization and results of experiment 1 are presented schematically on the left side of fig. 1. The species of coccidia that were represented by discharges of large numbers of oöcysts, namely, over 1,000 per drop from the

Fig. 1.—Extent of Treatment with Sulfaguanidine and Occurrence of Heavy Oöcyst Discharge Resulting from the Inoculations of Experiments 1 and 2.



Solid bars = Treatment; Abbreviations in open bars = Oöcyst discharge; Triangles = Inoculations. ALA = *E. alabamensis*; ELIPS = *E. ellipsoidalis*; AUB = *E. auburnensis*; ZUR = *E. zurnii*; BOV = *E. bovis*; D = Died; BUK = *E. bukidnonensis*; S = Sacrificed.

surface of the sugar flotation, and the weeks after inoculation in which they appeared are indicated by designated abbreviations placed in appropriate relation to the time scale. Sometimes a massive oöcyst discharge of a given species may require only a portion of the weekly period for which it is recorded. Blank spaces within the open bars in fig. 1 represent weekly periods in which the daily examinations either were negative or revealed only relatively small numbers of oöcysts.

The results may be summarized as follows:

1) Seven of the 8 calves discharged large numbers of *E. alabamensis* oöcysts during the second week of the experiment. The infections with this species had all subsided by the fourteenth or fifteenth day after inoculation.

2) The 4 untreated calves (144, 158, 160, and 162) developed severe clinical coccidiosis during the third week of the experiment The first 3 died on the twenty-first twenty-seventh and twentieth days, respectively, after inoculation. The fourth (162) was killed when moribund on the fifteenth day.

3) The fatal infections involved a mixture of species having prepatent periods of three and four weeks. The relative importance of the different species at any given point in such mixed infections is obviously difficult to determine, but *E. bovis* appeared to be the most important single species involved in each of the clinical infections, as it exceeded all the other species in both oöcyst production and tissue invasion. However, large numbers of *E. zurnii*, *E. bukidnonensis* and *E. auburnensis* were discharged by 1 or more of the untreated calves, indicating that these species were probably of some importance in all the infections. In the case of *E. auburnensis*, which has a long prepatent period, death of the host during the third week would preclude the possibility of demontrating its oöcyst discharge. Similarly, the calf killed on the fifteenth day (162) had not lived long enough to discharge *E. bovis* oöcysts, but numerous mature macrogametocytes of this species were demonstrated in stained sections of the colon.

4) The 4 treated calves (145, 157, 159, 163) failed to develop clinical coccidiosis and none died.

5) Only small numbers of oöcysts were discharged by the treated calves during the third and fourth weeks after inoculation; frequently the flotations were negative.

6) Two of the treated calves (145 and 163) passed large numbers of *E. bovis* oöcysts during the fifth week of the experiment, approximately two weeks after treatment had been discontinued. Oöcyst production thus appears to have occurred two weeks later than is normal for this species. Reinfection from the pen mate was possible in 1 case (145) but was unlikely in the other (163), because the pen mate (162) had not discharged *E. bovis* oöcysts before it was killed.

Experiment 2.—This experiment had two purposes, namely, (1) to check the results of experiment 1, which indicated that sulfaguanidine failed to protect the host against *E. alabamensis*, and (2) to test the resistance, to heavy inoculations of *E. alabamensis*, of hosts that had experienced previous infection with this species while receiving sulfaguanidine.

Five calves were used in this experiment. Four of these were the treated calves (145, 157, 159, and 163) of experiment 1; the fifth was an unpaired calf (151), which had been inoculated along with those of experiment 1 and differed only in having received 10 doses of sulfaguanidine before the inoculation. It had failed to discharge oöcysts of *E. alabamensis* as the result of the inoculation (*see* fig. 1).

On Aug. 9, 10, and 11, 1941, all 5 calves were inoculated with oöcysts, about 200 million per calf, collected from several calves of experiment 1 during the second week, at which time *E. alabamensis* comprised over 95 per cent of the oöcyst discharge.

Sulfaguanidine treatment was discontinued five days before inoculation in 3 cases (145, 159, and 163) and was continued without interruption for seven days after inoculation in the other 2 (157 and 151).

The organization and results of experiment 2 are shown schematically on the right side of fig. 1.

The results may be summarized as follows:

1) All 5 calves developed a severe diarrhea within five days after inoculation. The feces were quite watery, yellowish in color, and possessed a characteristic acrid odor.

2) In all 5 calves, the infections resulted in a very heavy production of *E. alabamen-*

sis oöcysts. This appeared in the feces during the second week after inoculation in the case of the 4 calves which lived that long, and was demonstrated at *post mortem* in the calf which died on the eighth day (163).

3) Two calves (159 and 163) of the 3 receiving no drug died on the fourteenth and eighth days, respectively, after inoculation. The death of 1 (159) was due directly to the *E. alabamensis* infection, no other species being involved. The death of the other (163) may have been complicated by the presence of *E. bovis*. By the time the host died, however, the latter species had disappeared.

4) The 2 calves (157 and 151) receiving extended treatment with sulfaguanidine survived.

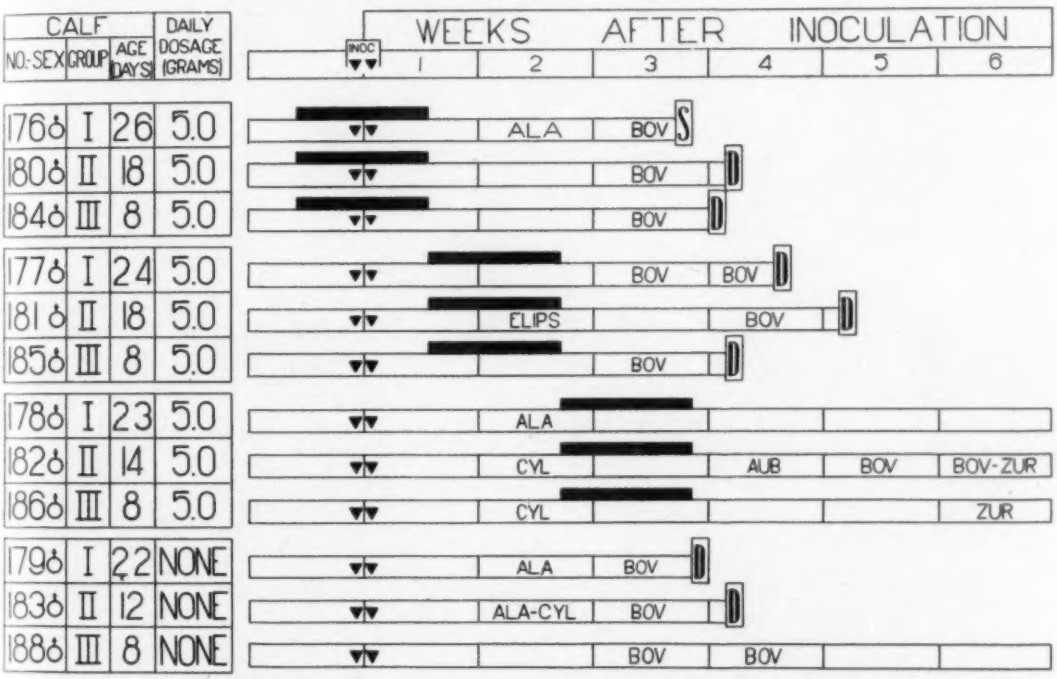
Experiment 3.—This experiment was designed to test the efficacy of sulfaguanidine against severe *E. bovis* infections. The drug was administered for specified short intervals prior to the onset of severe symptoms.

Twelve male calves, ranging in age from 8 to 26 days, were divided into 3 groups. Each group of 4 calves was assigned to one double isolation house. Special precautions were taken to avoid reinfection. All calves in the group were transferred together, twice daily, to a thoroughly cleaned compartment. The chance of picking up sporulated oöcysts was thereby reduced to a minimum, if not altogether eliminated.

On Aug. 26 and 27, 1941, all calves were inoculated with equivalent portions of a suspension in which *E. bovis* oöcysts, derived from the clinical cases of experiment 1, were abundant. Differential counts gave the following approximate proportions: One-third, *E. bovis;* one-third, *E. alabamensis;* one-third, other species. Each calf received approximately 50 million sporulated oöcysts.

In this experiment, 5 Gm. of sulfaguanidine were given daily for specified eight-day periods to 3 calves of each group. The drug was suspended in a small amount of water and fed by nipple approximately thirty minutes before the morning meal. The eight-day treatment was begun three days before inoculation on the first calf of each group, five days after inoculation on the second, and thirteen days after inoculation on the third; no drug was given to the fourth calf of each group.

Fig. 2.—Extent of Treatment with Sulfaguanidine and Occurrence of Heavy Oöcyst Discharge Resulting from the Inoculation of Experiment 3.



Solid bars = Treatment; Abbreviations in open bars = Oöcyst discharge; Triangles = Inoculation. ALA = *E. alabamensis;* ELIPS = *E. ellipsoidalis;* AUB = *E. auburnensis;* ZUR = *E. zurnii;* BOV = *E. bovis;* D = Died; CYL = *E. cylindrica;* S = Sacrificed.

The organization and results of experiment 3 are presented schematically in fig. 2.

The results may be summarized as follows:

1) *E. alabamensis* oöcysts were discharged during the second week by 4 of the 12 calves. The infections with this species probably played no significant part in the development of the subsequent clinical infections. However, their occurrence in relation to host age is of incidental interest. Three of the 4 calves showing *E. alabamensis* belonged to the oldest group (group I) and the fourth to the intermediate group (group II); none of the 8-day-old calves (group III) responded to the inoculation with *E. alabamensis*. This result is in agreement with the observation that very young calves seldom show natural infections with this species, in spite of the fact that its prepatent period is short.

2) The 3 calves (176, 180, and 184) receiving early treatment developed severe *E. bovis* infections during the third week. One calf (176) was killed on the twentieth day, when it was moribund; the others died on the twenty-third and twenty-second days, respectively, after inoculation.

3) The 3 calves (177, 181, and 185) receiving treatment beginning five days after inoculation developed severe *E. bovis* infections during the third or fourth week. They died on the twenty-sixth, thirtieth and twenty-third days, respectively, after inoculation.

4) The 3 calves (178, 182, and 186) receiving treatment beginning thirteen days after inoculation did not develop *E. bovis* infections during the third or fourth week; all survived. They failed to discharge large numbers of *E. bovis* oöcysts during the third and fourth weeks. One calf (182) showed high counts of this species after the fourth week.

5) The 3 untreated calves (179, 183, and 188) developed severe *E. bovis* infections during the third week. Two calves (179 and 183) died on the twenty-first and twenty-third days, respectively, after inoculation.

DISCUSSION

Interpretation of the results of the foregoing experiments requires a consideration of the following points: (1) the apparent difference in the response of the 2 principal species of coccidia involved; (2) the distinctive features of the life cycles of these 2 species; and (3) the relation between the number of coccidia introduced and the resulting infection.

The two species of coccidia that are of most interest in the present work are *E. alabamensis* and *E. bovis*. In experiment 1, the infections involved a number of different species. Reasons have been given already for considering *E. bovis* the dominant factor and *E. alabamensis* of comparatively little importance in producing the fatal infections. In experiment 2, *E. alabamensis* was without doubt the important species. In experiment 3, *E. bovis* produced the pathological conditions and caused the deaths of the hosts, the relatively small numbers of other species present being of little consequence.

Marked differences between treated and untreated calves inoculated with *E. alabamensis* were not obtained. In experiment 1, both treated and untreated calves developed infections with this species. There appeared to be a tendency for the treated animals to discharge fewer oöcysts. Oöcyst production, however, was sufficiently great to indicate that the reproduction of a considerable portion of parasites introduced at the time of inoculation was not curtailed. Treatment in this experiment was begun two days after inoculation, at which time the asexual reproduction was presumably underway, and was continued beyond the period of oöcyst discharge. In experiment 2, likewise, both treated and untreated calves developed *E. alabamensis* infections, which in this case were severe and produced a characteristic diarrhea. The oöcyst discharge of the treated animals was as great as that of the untreated, enormous numbers being discharged in all the infections as the result of the exceedingly large inoculation. The death of 2 out of 3 untreated, as against none out of

2 treated calves, may indicate, however, that treatment caused the infections in the latter to be relatively less severe. Treatment in this experiment extended from before inoculation through the period of asexual reproduction. In experiment 3, the response of the untreated calves to the inoculation with *E. alabamensis* was not sufficiently clear-cut to warrant a comparison between treated and untreated animals.

On the other hand, sharp differences between treated and untreated animals inoculated with oöcysts of *E. bovis* were obtained, indicating that sulfaguanidine, under the conditions of the experiments, was more effective against *E. bovis* than against *E. alabamensis.* In experiment 1, the untreated calves developed fatal infections; the treated calves did not become sick, and they exhibited either a negligible or a much delayed and curtailed oöcyst discharge. Treatment in this experiment was begun two days after inoculation and was continued for at least twenty-one days. In experiment 3, the short treatment begun late in the incubation period of the disease just prior to the onset of severe symptoms in the control group produced a similar sharp difference between the group so treated and those treated earlier or not at all.

E. alabamensis is a rapidly reproducing species that completes its life cycle in eight to twelve days. Its asexual stages develop in the epithelial cells of the free ends of the villi of the small intestine. The merozoite family is small, not over 16 (often fewer) merozoites being produced by each schizont. The sexual stages likewise develop in the epithelial cells of the villi of the small intestine. Groups of parasitized villi appear as white patches protruding above the surface of the mucosa. Natural infections with this species are seldom, if ever, seriously harmful to the host. The observations from which this brief description is drawn will be discussed more fully in another paper.

E. bovis has a prepatent period of approximately twenty days. Asexual reproduction—at least that portion of it which is known—takes place by means of relatively large schizonts visible to the naked eye.[8] These are most abundant in the small intestine, where they lie imbedded in the capillary network in the free ends of the villi just beneath the intact epithelium. The merozoite family is very large, thousands of merozoites being produced by each schizont. The sexual stages develop in the epithelial cells of the large intestine. This species is often one of the principal forms involved in clinical coccidiosis as it occurs on the dairy farm.

Massive reproduction of coccidia within the host tissues is culminated, after an apparently limited number of asexual generations, by oöcyst production. Since the number of parasites produced each generation is also limited, it follows that the extent of invasion at the end of the parasitic multiplication is in direct proportion to the number of parasites gaining entrance into the host. In other words, the severity of an infection with a given species is a function of the number of sporulated oöcysts ingested. Due to differences in the life cycles of the various species, such as exist between *E. alabamensis* and *E. bovis,* one would expect to find differences in total reproductive capacity and degree of tissue destruction. The minimum number of infective oöcysts required to produce clinical coccidiosis would not necessarily be the same for any two species. Interpretation of experiments with a single species and comparisons between species must take into account the relative numbers of coccidia given in the test inoculations.

If a treatment fails to destroy or completely repress all the coccidia present in an infected animal, its effectiveness against clinical coccidiosis must depend upon its ability to keep the parasite population below the level at which host tissue is severely damaged. The present experiments indicate that sulfaguanidine in the doses given did not destroy all the coccidia. This was particularly evident in the case of *E. alabamensis* at the two levels of infection tested in the first and second

[8]Boughton, Donald C.: An Overlooked Macroscopic Intestinal Lesion of Value in Diagnosing Bovine Coccidiosis. N. Am. Vet., 23 (1942), pp. 173-175.

experiments. The heavier infection (experiment 2) reached a level damaging to the host. Whatever destructive effect the drug may possess against *E. alabamensis*, it was not sufficient, in the dose used, to protect the host against a massive infection. In the case of *E. bovis*, even when sulfaguanidine protected the calves from fatal infections, a few of the parasites succeeded in completing the life cycle. In the third experiment, the drug was effective only when given late in the incubation period of the infection, at which time the reproductive potential is presumably the greatest. This indicates that the drug, through its retarding effect, prevented the development of a large proportion of the organisms and thus kept the final parasite population below the lethal level. Treatment earlier in the incubation period was not effective. This suggests that the coccidia which were not affected by the drug were able, upon discontinuance of treatment, to reach the period of stepped-up reproduction, and thus, even though their original numbers may have been reduced, to produce a population sufficiently large to kill the host. It is possible, of course, that for some reason the drug is relatively more effective against the later generations than against the early ones. It should be pointed out that as long as the treatment fails to rid the intestine entirely of *E. bovis*, it is theoretically possible that infections with this species considerably heavier than those produced in the present study would not be controlled by treatment. If one is dealing with relatively low-grade infections, such as those studied by Foster, Christensen, and Habermann[5] in lambs, treatment may appear to be 100 per cent effective simply because the parasite population in the treated animals drops below the level at which it can be demonstrated by the technique employed.

The data reported here do not provide an explanation of the mechanism of the action of sulfaguanidine. However, the difference in the response of *E. alabamensis* and *E. bovis*, when considered in the light of their respective life cycles, is of possible significance. The exposure of merozoites of *E. bovis* to the contents of the intestinal lumen is almost certainly greater than that of the merozoites of *E. alabamensis*. The bursting of the large schizonts of the former species liberates an enormous swarm of merozoites. The release of *E. alabamensis* merozoites from their small schizonts is probably accomplished with less violence and is conceivably followed by an almost immediate penetration of nearby host cells. Since the sexual stages of *E. bovis* are often restricted to the large intestine, it is certain that one asexual generation must pass through the ileocecal valve. Sulfaguanidine is known to be absorbed from the intestinal contents rather slowly. The longer exposure of *E. bovis* merozoites to the lumen contents may give the drug a better opportunity to act against *E. bovis* than against *E. alabamensis*.

SUMMARY AND CONCLUSIONS

Three experiments are reported in which experimental infections of bovine coccidiosis were treated with sulfaguanidine.

Favorable results were obtained in the case of *Eimeria bovis* infections when the drug was given daily (*a*) at the rate of 0.1 Gm. per kg. of body weight for a three-week period beginning two days after inoculation; and (*b*) in 5-Gm. doses for an eight-day period beginning thirteen days after inoculation.

Treatment of *E. bovis* infections for eight-day periods begun three days before and five days after inoculation was not effective.

Marked differences between treated and control infections were not obtained in the case of *Eimeria alabamensis*.

The relation between successful treatment and size of coccidial population is discussed.

Distinctive features of the asexual stages of *E. alabamensis* and *E. bovis* are pointed out; their possible significance in the present work is suggested.

It is concluded that sulfaguanidine gives promise of being useful against certain types of bovine coccidiosis.

The Fowl Tick (Argas Persicus), a New Vector of Anaplasmosis

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SOON AFTER ANAPLAMOSIS was recognized as a separate disease, ticks were incriminated as vectors. At the present time, 18 species have been shown to transmit the disease experimentally. Of these ticks, all but one belong to the family Ixodidae. Only Rastegaieff,[1] while working in Russia, has incriminated an Argasine tick, *Ornithodoros lahorensis* Neumann, as a vector of anaplasmosis. Boynton, Herms and Howell (unpublished data) were unable to obtain transmissions by means of *O. coriaceus* or *O. (Otobius) megnini*. The authors have failed to find additional published data on anaplasmosis transmission by ticks belonging to the family Argasidae.

In the more arid area of the southwestern part of the United States, cattlemen have frequently inquired about the rôle played by the spinose ear tick, *O. megnini* (Dugès), and less frequently the fowl tick, *Argas persicus* (Oken), in spreading anaplasmosis. In order to answer these questions, the following experiments were performed, designed to test both the mechanical, stage to stage, and hereditary transmission of the Argasine ticks which are most frequently encountered in the Southwest.

MATERIALS AND METHODS

All transmission experiments were conducted in isolation units of a barn designed and constructed for experiments in the transmission of anaplasmosis. All ticks and flies were rigidly excluded from this barn. The cattle used in the experiment were kept in the barn at least forty days before use, to minimize the chance of accidental infection before the animals were used experimentally. As an additional precaution, the cattle were purchased from the college dairy herd where no cases of anaplasmosis has been recorded.

The fowl ticks used were collected from their hiding places in and around chicken houses. As a result, no larvae nor first stage nymphs were collected; the other stages were represented in about equal numbers. The spinose ear ticks were taken from the ears of healthy cattle for stage to stage transmission. For the hereditary transmission trial, mature nymphs were removed from an animal sick with anaplasmosis and allowed to molt and lay eggs in the laboratory. The larvae from these eggs were then used.

Feeding was accomplished in one of two ways. Small sacks of durable material, slightly larger than the ear to which they were to be attached, were made. The opening was lined with lamb's wool to prevent the escape of small ticks and to make it more comfortable for the cow when the sack was attached to her ear. Ticks were fed either in the ear or in small metal boxes on the back, as described in The Public Health Reports (1933), pp. 1081-82, with the exception that the box was glued on rather than secured with tape. By removing the screw cap, it was possible readily to remove or add ticks when desired.

EXPERIMENTAL PROCEDURE AND RESULTS

Experiment I.—About 75 fowl ticks collected at Chickasha, Oklahoma, May 16, 1940, and kept in the laboratory, were placed in the ear of cow 239 on May 31. This animal had an acute case of anaplasmosis. Two days later, June 2, these ticks were removed. It was apparent that 58 had fed. When the ticks were first attached, blood smears from cow 239 showed less than 5 per cent Anaplasma infested red blood cells; however, approximately 30 per cent were involved when the ticks were removed. The ticks were stored in the laboratory in small pill boxes at room temperature and humidity.

Two weeks later, June 15, the ticks which had fed on cow 239 were placed in the ear of cow 252, a healthy susceptible animal, and allowed to remain until the morning

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This paper reports a coöperative project conducted by the Oklahoma Agricultural Experiment Station and the Bureau of Animal Industry, U. S. Department of Agriculture.

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[1]Rastegaieff, A. F.: A new Vector in the Transmission of Hemoparasites of Domestic Animals, *Ornithodoros lahorensis* Neumann 1908. Ann. Inst. Pasteur, liv (1935), pp. 250-258.

of June 17, when it appeared that 38 had fed.

Blood smears were made from experimental cow 252 at weekly intervals for the first three weeks and thereafter twice a week. On August 15, smears were suspicious, and on August 17 definitely positive. Cow 252 developed a severe case of anaplasmosis with a temperature rise to 105.8 F., marked icterus, severe anemia, and inappetence. The cow was used as a source of infective blood for several months and was afterward sold for slaughter.

Experiment II.—On Sept. 19, 1941, 103 fowl ticks from a large group collected on July 23, at Omega, Oklahoma, were fed to partial repletion on the back of cow 263, which was in the initial stage of anaplasmosis and showed approximately 25 per cent of the erythrocytes involved. The time required for feeding varied from one to five minutes, depending upon the individual tick. When approximately one-third replete, the ticks were quickly transferred to cow 264, a nonimmune animal.

Blood smears from cow 264 were examined twice weekly for four months and revealed no marginal bodies. The susceptibility of this animal was later demonstrated by the injection of carrier blood which produced a mild case of anaplasmosis in 42 days.

Experiment III.—At the time the ticks in experiment II were fed to partial repletion on the acute case, cow 263, 300 other ticks were permitted to complete their feeding on the same animal. These ticks were pooled with the ticks used in experiment II and stored in the laboratory until October 21, when they were fed during the night on cow 246, a susceptible animal. It was believed that 284 ticks took some blood.

Regular examination of blood smears from cow 246 failed to show anaplasmal infection. She was later proved nonimmune, for she developed a mild case of anaplasmosis following the injection of 10 cc. of blood from a carrier animal.

Experiment IV.—Several hundred larval and nymphal spinose ear ticks, *O. megnini* (Dugès), were collected from cattle in Beaver County, Oklahoma, July 31. They were stored in the laboratory until September 10, when 50 were placed in the ears of cow 252, an animal just recovering from anaplasmosis transmitted by *A. persicus* (Oken) in experiment I. Two weeks later, 41 live ticks were removed and transferred to the ears of cow 243. Nine ticks were dead. The live ticks appeared to have fed and many had molted. The 41 ticks remained in the ears of cow 243 until October 7, when it was evident 37 had fed. On removal, most of them had molted; 4 were dead and apparently had not fed.

Experimental animal 243 was bled at regular intervals and microscopic examinations of the blood made with negative results. Subsequent inoculation with carrier blood proved this cow was susceptible.

Experiment V.—Twenty-one replete spinose ear tick nymphs were removed from the ear of an old range cow that had recently died of anaplasmosis. This animal had been shipped into Osage County, Oklahoma, from western Texas about five months previously.

The ticks from this cow molted to the adult stage between September 10 and 16, and copulation was noted September 17, 19, and 20. Egg deposition began October 4 and continued until December 16. Hatching began October 29 and continued until January 8. On this date, all of the larvae (approximately 2,000) were combined and placed in the ears of cow 244, to determine the possibility of transmission from parent to offspring through the eggs. Feeding began almost at once, and many ticks reached the last nymphal stage. Frequent blood examination of cow 244 failed to reveal any Anaplasma. This animal was later proven susceptible by the injection of carrier blood.

The above experiments are summarized in table 1.

DISCUSSION

The experiments reported here indicate that fowl ticks, *A. persicus* (Oken), may occasionally transmit anaplasmosis from sick to healthy animals. However, they are probably not an important factor. These ticks normally feed on fowls, but during the past two years, one of the authors (D.E.H.)

TABLE I—Summary of Transmission Experiments.

Exp. No.	No. of Experimental Animal	No. of Source Animal	% R.B.C.* of Source Animal Infected	No. Ticks Used	Time on Source Animal	Time on Experimental Animal	Time Between Feeding	Time Before Anaplasmosis Appeared	Type of Transmission Attempted
I.	252	239	5-30	38	2 days	2 days	16 days	61 days	Biological (Stage to stage)
II.	264	263	25.0	103	1 to 5 min.	10 hr.	1 min.	.....	Mechanical
III.	246	263	25.0	284	10 hr.	10 hr.	32 days	.....	Biological (Stage to stage)
IV.	243	252	28.5	37	14 days	13 days	6 hrs.	.....	Biological (Stage to stage)
V.	244	Field Case	36.0	About 2,000	Not Known	60 days	.....	.....	Biological (Hereditary)

*R.B.C. = red blood cells.

has received from county agents for identification three lots of fowl ticks which were reported to have been taken from cattle. This indicates that fowl ticks may feed on cattle more frequently than has been assumed, particularly since they usually feed at night when they would not be observed. Furthermore, one who has watched the willingness and rapidity with which they feed on cattle under experimental conditions can have little doubt that they may occasionally feed on cattle under natural conditions, especially if the favored host has been removed.

Spinose ear ticks are very common in much of the area where anaplasmosis is a serious problem, and cattlemen freqently request information concerning the rôle they play in the spread of anaplasmosis. Since these ticks normally feed on a single individual only, the infection would have to pass from parent to progeny through the egg to reach a second cow. There is little epidemiological and no experimental evidence to suggest that this occurs.

SUMMARY

In one out of three experiments, it was possible to transmit anaplasmosis by means of the fowl tick, *Argas persicus* (Oken). Efforts to transmit the disease experimentally by means of the spinose ear tick, *Ornithodoros megnini* (Dugès), were unsuccessful.

Effects of Pituitary Gonadotropins on the Ovaries and the Induction of Superfecundity in Cattle

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THE MARKED OVARIAN reaction to gonadotropic hormones so easily produced in certain laboratory animals has not resulted in their wide use for control of the reproductive cycle in animal breeding or as a therapeutic agent in veterinary medicine.

Zavadovskii, *et al.*,[1] used pituitary emulsions on a small number of cows and succeeded in inducing ovulation. Rumjancev[2] found that fresh pituitary substance did not induce heat and had no marked effect on the duration or intensity of estrus. Follicular growth with subsequent ovulation, atresia or luteinization and a considerable increase in the number of corpora lutea were noted in approximately half of the cases.

Two apparently shy-breeding heifers were treated by Asdell.[3] Both of them came came into heat later and were bred. One became pregnant and the other, after showing nymphomania for a time, also conceived.

Ovulation was produced with prolan in both open cows and heifers and in pregnant cows by Zavadovskii, *et al.*[4] They reported that many of them came into heat three to five days later, but those which were bred did not conceive. They also indicated that, in all their experiments, the artificial estrus did not disturb the natural estrual cycle. Their results with equine gonadotropin seemed to have been somewhat similar. Later, Zavadovskii and Eskin[5] conducted experiments to determine why ovulation induced by a gonadotropic agent is not followed by conception. They were unable to detect anything abnormal about the ova, but did point out that the corpora lutea which were produced experimentally were incapable of disturbing normal estrual rhythm. The appearance of the uterine mucosa, however, seemed to be normal for implantation. They suggested that the failure of conception was due to the fact that the female genital tract was not ready to receive the spermatozoa and that, therefore, their vitality and migration were below normal.

Hart and Cole,[6] from limited data, suggested that the minimal dose of equine gonadotropin for eliciting a response in cattle was approximately 750 rat units. They observed follicular rupture from this dosage and on larger dosages obtained excessive stimulation.

Prolan or prolan and folliculin were administered to cows eight days after the preceding heat period by Pavlov and Soldatenkov,[7] but very few of them came into

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Paper No. 305 from the Department of Genetics, Wisconsin Agricultural Experiment Station.

The authors wish to acknowledge the valuable aid of W. G. Venzke and A. V. Nalbandov, who assisted with parts of this study. The work was supported in part by a grant from the Alumni Research Foundation. Further acknowledgment is due to the assistance of personnel provided by the National Youth Administration and the Work Projects Administration.

[1]Zavadovskii, M. M., Eskin, I. A., and Ovsjannikov, G. F. (Lab. Phys. Devel., Inst. Anim. Breed., Moscow): Regulirovanie zenskogo polovogo cikla u korov. (The control of the female sexual cycle in cows.) Trud. Dinam. Razvit., *9* (1935), pp. 75-96, (English summary); cited from A.B.A., *4*, p. 37.

[2]Rumjancev, N. V. (Lab. Physiol. Lact., Inst. Anim. Breed., Moscow): Vlijanie totaljnogo preparata perednei doli gipofiza na polovye organy korov. (The influence of total prepituitary preparation on the genitalia of the cow.) Usp. zooteh. Nauk, *5* (1938), pp. 73-85, (English summary); cited from A.B.A. *8*, p. 130.

[3]Asdell, S. A.: Some Practical Applications of Endocrinology to Animal Husbandry. Proc. Am. Soc. Anim. Prod. (Nov. 1935), pp. 235-237.

[4]Zavadovskii, M. M., Eskin, I. A., and Ovsjannikov, G. F.: *Op. cit.*

[5]Zavadovskii, M. M., and Eskin, I. A. (Lab. Phys. Develop., Inst. Anim. Breed., Moscow): New Data on Regulation of the Sexual Cycle in the Cow. Bull. Biol. Med. Exp. USSR, *5* (1937), pp. 200-202; cited from A.B.A. 7, p. 122.

[6]Hart, G. H., and Cole, H. H.: The Gonad-Stimulating Hormone of Mare Serum. Proc. Am. Soc. Anim. Prod., (1933), pp. 262-268.

heat. The cows were inseminated but did not conceive.

Other reports on the use of prolan in the treatment of ovarian hypofunctions, sterility, and nymphomania give little information on the specific effects of the hormone treatment, so will not be discussed.

NATURE OF STUDY

The primary objective of this study was a determination of the effect of pituitary gonadotropins upon bovine reproductive functions. More specifically, observations were made upon the ability of gonadotropins to produce follicular maturation, estrus, ovulation, and the formation and maintenance of corpora lutea. Tests were also made to determine whether eggs from artificially matured and ovulated follicles were potentially fertile. In addition, the effects of treatment upon the rhythm of the natural estrual cycle were noted.

Different types of extracts of sheep pituitary glands were studied—unfractionated, follicle-stimulating, and luteinizing. In a few cases, unfractionated extracts from pituitary glands of other species were used. The various extracts were administered by different routes. Some were given subcutaneously; others intravenously; and in many cases, a combination of the two methods of administration was used.

The animals were classified according to the stage of the reproductive cycle at the time treatment was begun. The ability of these different kinds of animals to react to gonadotropin was observed, as well as the effects that the stage of reproductive cycle might have on qualitative results obtained.

Other problems that received consideration were the tendency of gonadotropic treatments to result in production of antigonadotropic hormone and also the ability of a nonspecific augmenter to enhance the effectiveness of pituitary gonadotropins in cattle.

MATERIALS AND METHODS

Time and Duration of the Study.—This work was done during the interval of 1934 to 1941, inclusive. Small numbers of animals were available at any one time, often only one or two. The facilities for maintaining animals also were limited, so that, in some cases, months intervened between animals or treatments that were to be compared.

Experimental Animals.—The cattle used in these experiments were primarily of dairy breeding (mostly Holsteins), and are grouped as calves, normal cows, and cows with incomplete or questionable breeding histories.

The calves may be classed as "vealers." The weights ranged from 94 to 190 lb., and in so far as ages were known (only on 24 calves), from 17 to 117 days. Some of the calves were raised at the University farm, either in the dairy herd or in the experimental herd, whereas others were purchased from local stockyards.

The normal cows were ones for which there seemed to be no reason to question their ability to reproduce. This meant that they either were pregnant at the time of treatment or had recently calved, and frequent palpations of the genital organs showed no abnormalities which would be likely to interfere with reproduction. Most of these cows were purchased from farmers as "springers." If not treated while pregnant, they were permitted to calve in the experimental herd and frequent observations were made on them until the time of treatment. Some of the cows came from the University herd, but these were ones for which an equal amount of information was available.

The cows with incomplete or questionable breeding histories came either from the University or from other state institution herds. These cows were being discarded, usually, for reasons other than extremely low breeding efficiency. Many of them, however, had been bred one or more times without conceiving. From the responses obtained with these animals, there seems to be no very good reason for putting them in a different group from the normal cows. It has been done on the possibility that they might represent a selected population.

Gonadotropic Extracts.—During the first few years, the endocrine preparations could be made in small quantities only, usually enough for one to three animals. Bioassay control was limited in the early stages of the work; thus, there was undoubtedly variation among the different preparations which was not kept constant in terms of physiological units determined on laboratory animals. Both of these conditions were materially improved during the last five or six years of the study when extracts were prepared in larger quantities and were more carefully controlled in the chemical and bioassay laboratories.

The gonadotropic extracts, unless otherwise stated, were prepared from acetone desiccated

[7] Pavlov, G. N., and Soldatenkov, P. F.: Experiments on Inducing Heat in Cows by Folliculin and Prolan. Proc. Leningr. Inst. Dairy Cattle Breed., 1, (1933) pp. 115-120; cited from A.B.A., 2, p. 107.

sheep pituitary gland powder. They may be classified as follows: (1) unfractionated (Unf.), prepared either as a 50 per cent pyridine-33 per cent acetone-water soluble extract, or as a simple aqueous extract of the original powder. Such preparations usually produced extensively luteinized ovaries in immature test rats; (2) follicle-stimulating extract 1 (FS-1), prepared by the method of Fevold, *et al.*[8] The preparations made by this method were inadequately tested on rats, but slight luteinizing activity was displayed in the few tests that were run; (3) luteinizing extract 1 (Lut-1), prepared by the method of Fevold, *et al.*[8] None of these preparations was tested on rats; (4) follicle-stimulating extract 2 (FS-2), prepared by the method of McShan and Meyer.[9] Each of these preparations was assayed, using 0.5-Gm. equivalent dosages, on 21-day-old rats. For 3 to 6 animals, the average ovarian weight was usually about 60 mg., but ranged from 25 to 90 mg. Little or no evidence of luteinization was observed in the test rats' ovaries when they were killed after 9 semidaily injections; (5) luteinizing extract 2 (Lut-2), prepared by a procedure developed by McShan (unpublished). These preparations elicited no follicle stimulation or luteinization in immature rats when administered alone in 0.5-Gm. equivalent dosages. They did, however, produce extensive luteinization when 0.5-Gm. equivalent was combined with 0.5-Gm. equivalent of test follicle-stimulating extract and administered to 21-day-old rats for 9 semidaily injections.

The dosages have been expressed in Gm. equivalents. This refers to the number of Gm. of the original acetone desiccated powder which had been extracted and were represented in the dosage. This method has been used rather than expression in terms of rat units because of incomplete bioassay information.

METHODS OF TREATMENT

The quantity of extract to be injected was dissolved in distilled water. As a rule, 5 cc. of fluid was used and injections were made once daily. Subcutaneous injections were made in the neck or shoulder regions, and intravenous injections were made into either jugular vein. All extracts were stored in a refrigerator.

DATA AND DISCUSSION

Preliminary Experiments with Calves.—Twenty-six calves were used in the preliminary trials with pituitary gonadotropins. The detailed data are not included here, but the interpretations which the authors have made of the results are as follows:

The ability of the ovaries to show a measurable gross response to treatment with gonadotropins appeared to be dependent upon the presence in the ovaries of vesicular follicles. Further than this, however, the increase in size of ovary bore only a slight association with the number of vesicular follicles present at the beginning of treatment or their size. The superficial follicles with antrums could be destroyed by electrical cautery and the ovaries still showed a marked follicular development from gonadotropic treatment. From this, it was inferred that the ability to respond was indicated by the presence of vesicular follicles, but was not directly dependent upon it.

The formation of corpora lutea from subcutaneous injections of unfractionated extracts seemed to be associated more closely with the interval of time during which the hormone was acting than it did with the ovaries or the follicles having obtained a certain minimum size. No corpora lutea were observed after six days of treatment, but were found after eleven or twelve days of treatment. This was true even though many of the six-day animals showed larger ovaries and larger follicles than were present in the eleven- or twelve-day animals.

Various attempts were made to increase the follicle-stimulating effectiveness of the gonadotropic extracts. Administration by repeated intravenous injections appeared to be much less effective than by repeated subcutaneous injections. Administration of a mixture of hemin and gonadotropic extract was no more effective than administration of the extract alone. Also simultaneous injection of an estrogen (theelin) did not appear to enhance or modify the ovary-stimulating ability of the gonadotropin.

Comparison of unfractionated extracts of beef and sheep pituitary glands indicated the former to be only slightly less effective than the latter when injected into calves. This is at variance with the apparent wide difference in the potencies of these extracts when injected into rats.

[8]Fevold, H. L., Hisaw, F. L., Hellbaum, A., and Hertz, R.: Sex Hormones of the Anterior Lobe of the Hypophysis. Am. J. Physiol., *105* (1933), pp. 710-723.

[9]McShan, W. H., and Meyer, Roland K.: The Preparation and Properties of Pituitary Follicle-Stimulating Fractions made by Trypsin Digestion. J. Biol. Chem., *135* (1940), pp. 473-482.

Calves Treated by Subcutaneous Followed by Intravenous Injection of Pituitary Gonadotropins. — Pituitary gonadotropins have been used in the induction of ovulation in laboratory animals by various workers. Relatively few investigations have been made on farm animals. The manipulation of dosages and injection methods for different combinations of follicle-stimulating and luteinizing extracts appeared from these studies to be important considerations.

Primary emphasis in the present study was placed on the testing of combinations of pituitary materials and combinations of injection methods for production of ovulation. Thirty calves were treated by administration of follicle-stimulating extracts subcutaneously followed by intravenous injection of either a luteinizing extract or follicle-stimulating extract (table 1).

TABLE 1.—Calves Treated by Subcutaneous Followed by Intravenous Injections of Pituitary Gonadotropins.

	Subcutaneous Injections			Intravenous Injections							
Animal No.	Extract Type and No.*	Dosage—Gm. equivalents	No. of Injections	Extract Type and No.*	Dosage—Gm. equivalents	No. of Injections	†Days before observation	Ovarian wt.—Gm.	No. of corpora lutea	Diam. of largest follicle—mm.	No. of eggs found
3	(FS-1)3	6	6	(Lut-1)4	10	2	8	15	2	20	2
4	(FS-1)3	6	6	(Lut-1)4	10	2	11	6	0	23	—
5	(FS-1)2	15	6	(Lut-1)4	10	2	9	9	6	17	4
6	(FS-1)3	2	2	(Lut-1)4	8	1	6	3	1	8	0
9	(FS-1)8	1	1	(FS-1)8	2	2	3	1	0	7	—
10	(FS-1)8	2	1	(FS-1)8	4	2	3	4	0	11	—
11	(FS-1)8	4	2	(FS-1)8	6	3	5	3	0	9	—
21	(FS-1)10	15	6	(Lut-1)11	3	2	8	2	0	13	—
156	(FS-2)104CS	3	1								
	(FS-2)107	6	2	(FS-2)107	3	1	7	26	0	21	—
162	(FS-2)110WS	4.5	3	(FS-2)110WS	1.5	1	10	16	3	Unk.	—

†For explanation of abbreviations, *see* p. 78. ‡Interval calculated from beginning of treatment.

The first calf (3) treated by this method showed the presence of 2 corpora lutea when autopsied at eight days after the beginning of treatment. Two eggs were recovered by flushing the oviducts with physiological saline. With the demonstration of a method for production of ovulation, a second animal (4) was placed on the same treatment and then artificially inseminated. Three additional subcutaneous injections of luteinizing extract, totaling 3.6-Gm. equivalents, were given after the intravenous treatment. This was done because it was suspected at the time that further administration of a luteinizing extract might enhance the development of corpora lutea in the case of pregnancy. As indicated by the work of Bunde and Greep[10] and Astwood and Greep[11], this now seems doubtful. For some reason, ovulation did not occur in this animal, but there was development of a 23-mm. follicle or cyst. Consequently, no test of the fertilizability of calf eggs was furnished by this experiment.

Calf 5 was treated in a manner similar to the 2 previous calves, but the extract used was prepared from sheep pituitary rather than from horse pituitary gland, as was the case of extract (FS-1)3. This calf also was artifically inseminated with semen collected from the vagina of a cow which had just been served by a bull. Again, 1 subcutaneous injection of luteinizing extract, 1.25-Gm. equivalents, was administered following the intravenous injection. Six corpora lutea were present in the ovaries at the time of autopsy, nine days after the beginning of treatment. Flushing of the uterine horns with physiological saline yielded 3 eggs, and 1 egg was recovered by tubal irrigation. This last ovum showed 1 large and 2 small cytoplasmic masses inside the zona pel-

[10]Bunde, C. A., and Greep, R. O.: Suppression of Persisting Corpora Lutea in Hypophysectomized rats. Proc. Soc. Exp. Biol. Med., *35* (1936), p. 235.

[11]Astwood, E. B., and Greep, R. O.: A Corpus Luteum-Stimulating Substance in the Rat Placenta. *Ibid.*, *38* (1938), p. 713.

lucida. These bodies certainly did not appear as normal blastomeres and it was left undecided whether fertilization had occurred in this egg.

The fact that 3 eggs were recovered from the uterine horn in calf 5, slaughtered at nine days, suggested that ovulation must have occurred by six days after the beginning of treatment, that is, assuming that a minimum of three days is required for transport of eggs through the oviduct. Calf 6 was given a shorter treatment with follicle-stimulating extract and the dosage, again using extract (FS-1)3, was reduced below that used with previous animals. This animal was slaughtered six days after the beginning of treatment, and at that time there was 1 corpus luteum in the ovary. However, search of the oviducts and horns of the uterus by flushing with saline failed to yield any eggs. It is possible that the corpus luteum was one with an entrapped ovum, although there was a definite stigma present. As pointed out in connection with the preliminary trials, no trace of corpora lutea formation was observed in animals slaughtered six days after beginning of treatment by subcutaneous method only. The results with the first 4 calves treated with the combination of extracts and injection methods suggested that such a procedure gave greater control over the production of ovulation than treatment by subcutaneous injection alone.

Observations early in the studies with calves indicated that there was a tendency for the gonadotropic hormone to produce approximately equal growth in a large number of follicles rather than to select a few follicles and produce a larger amount of growth in them. Introduction of an ovulation stimulus such as the intravenous injection of luteinizer tended, therefore, to cause ovulation in more than one follicle. This would be desirable in a litter-bearing animal, but in cattle the production of a single egg at a time would seem the logical goal.

Extract (FS-1)8, prepared from sheep pituitary glands, was one that had been used in ovulation studies of lambs and anestrual ewes. As reported by Casida[12], when small doses of this preparation were injected subcutaneously into 3- to 5-month-old lambs, follicular stimulation, ovulation, and formation of corpora lutea resulted. When administered in large doses, however, only marked follicular maturation was produced. Injection of the preparation one or two days subcutaneously, followed by one to three days intravenously, produced ovulation in the anestrous ewe. The number of ovulations produced in these trials were usually 1 or 2 per animal. This extract was used on calves 9, 10, and 11 to attempt ovulation with a minimum degree of ovarian stimulation and also to get data for comparison of the calf with the sheep. Dosages of 1-, 2-, and 4-Gm. equivalents were administered subcutaneously in 1 or 2 injections. These were followed by dosages of 2-, 4-, and 6-Gm. equivalents administered in 2 or 3 intravenous injections. No ovulations were produced and again a number of follicles were stimulated to a slight degree rather than just a few to a greater degree.

In each of the first 7 calves presented in table 1, the dosage of extract given by intravenous injection in terms of Gm. equivalents usually equalled or exceeded the dosage administered subcutaneously. Calf 21 was treated in such a way that the luteinizing extract in terms of Gm. equivalents was only one-fifth that of the (FS-1) extract administered subcutaneously. It constituted a further test of different ratios between the subcutaneously and intravenously administered preparations, with the idea that there might be an optimum ratio. No corpora lutea were formed from the treatment, however, within eight days. A 13-mm. follicle, which was the largest, was showing considerable vascularity. It is presumed that the dosage of luteinizing extract was too small to bring in the required ovulation stimulus.

Approximately four and a half years intervened between the treatments of the first 8 calves and the treatments of calves 156 and 162. During the meantime, methods for the preparation of more highly purified follicle-stimulating extract had been developed. The (FS-1) extracts used in the

[12]Casida, L. E.: Ovarian Stimulation in Immature Farm Animals. Proc. Am. Soc. Anim. Prod., (1934), pp. 144-145.

earlier years were in reality only partially purified. As noted earlier, the test rats which were treated with these extracts often showed considerable evidence of luteinization in their ovaries. The newer preparations were ones that gave little or no evidence of luteinization in rat ovaries.

Calves 156 and 162 were treated by subcutaneous followed by intravenous injections, both with the same type of extract, (FS-2). On the assumption that luteinizing activity was absent from these extracts but was needed to produce corpora lutea, these animals constituted a test of the effect of ovarian development upon the luteinizing action from the calves' own pituitary glands. It might be interpreted that this is what happened in calf 162, which formed 3 corpora lutea as a result of the treatment. No search was made for eggs in this animal.

This result could also be interpreted that luteinizing action is not necessary for the production of ovulation. It seems more conservative to presume, however, that even in the suckling calf, the pituitary gland is beginning to exert some gonadotropic activity and will initiate luteinizing action when subjected to ovarian activity. Further work should be done on this point using hypophysectomized calves. For the present, it is probably best to assume, always, that the animal's own pituitary gland may complicate the response obtained from any gonadotropic hormone treatment.

A total of 6 calves (not included in table 1) were treated subcutaneously for four days with 6-Gm. equivalents of the best follicle-stimulating extracts obtainable, followed two days later by intravenous treatments with 5-Gm. equivalents of a purified luteinizing extract of known potency, as determined by augmentation of follicle-stimulating preparations in rats. This treatment of the calves was presumed to produce ovulation, but no direct observations were made. The calves were artifically inseminated with a good quality of semen, collected with an artificial vagina from bulls of known fertility. The trial was to constitute a test of the potential fertility of eggs from artificially matured and ovulated follicles.

No further treatment was given these calves until twenty-three days later, when 2 of them were laparotomized to determine the condition of the corpora lutea and the possible presence of pregnancies. Seven corpora lutea were present in 1 and 18 in the other. Those in the latter calf were quite well-developed and were judged superficially to be functional. Those in the former were probably less well-developed or were showing some regression. One of the calves died four days after the operation and there was an opportunity to explore the uterus thoroughly for any contents. None were found. One horn also was removed from the other calf at the laparotomy and no contents were found in it.

Although there was no evidence of pregnancy at the laparotomies, nothing further was done with any of the calves for another seventeen days when the remaining ones were slaughtered. None of the calves showed evidences of corpora lutea at the time of slaughter, although 1 animal had shown them seventeen days earlier, as determined by laparotomy. There was no evidence that a pregnancy had ever occurred in any of these calves. Although it is not known definitely that ovulation had occurred in all of them, it is presumed that it had in most of them and that the corpora lutea had regressed. Because of the conditions observed in 2 calves on the twenty-third day, it is supposed that corpora lutea regressed between then and the fortieth day. It would appear that some effect of treatment could still be detected at autopsy, inasmuch as the ovaries averaged 3.6 Gm. in weight and the diameters of the largest follicles averaged 10.4 mm.

Fourteen other calves (also not included in table 1) were treated in essentially the same manner as the ones just discussed, including artificial insemination, but were slaughtered within four days after the intravenous injection. In addition, Progynon B (1,000 rat units) was administered to 3 of the calves two days before gonadotropin treatment and to 3 others on the day that luteinizing extract was given. Corpora lutea were present in 10 of the 14 calves and eggs were recovered from 8 of the 10.

Twenty-nine corpora lutea were observed, but only 15 eggs were recovered. Twelve of these were obtained from the oviducts and 3 from the uterine horns. One egg obtained from the oviduct of a calf receiving estrogen on the day of the luteinizing treatment appeared to contain 3 blastomeres, 2 of them each approximately half the size of the third. This probably was evidence of fertilization. Another egg recovered from a uterine horn (calf 201, not receiving

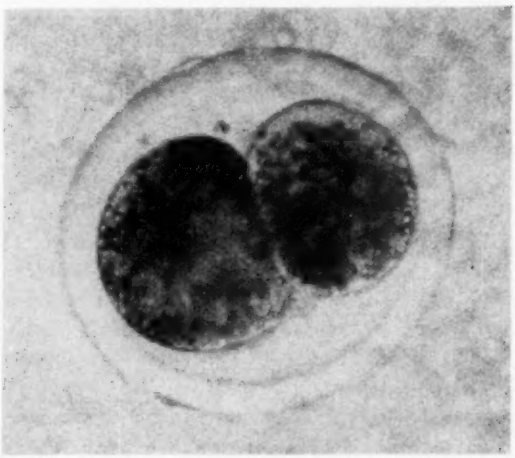

Fig. 1.—Two-Cell Embryo from the Uterine Horn of Calf 201. Approximately x300.

estrogen) appeared to be a normal 2-cell embryo. There seems to be no reason for questioning fertilization of this egg. An egg recovered from the uterine horn of another calf showed a herniated zona pellucida with very little cytoplasm enclosed. The remaining eggs appeared normal, with the possible exception of one completely enclosed in corona radiata cells. It may be that this was from a very recent ovulation, and that the corona cells are not shed until after ovulation.

The ovaries from 1 of these calves weighed 122 Gm. and were the heaviest ones produced in calves. The average ovarian weight for normal veal calves, reported by Casida, Chapman, and Rupel[13], was 1.9 Gm. The maximum response in calves, therefore, would appear to be an increase of about sixty-fold.

In general, the ovarian weights showed an extreme amount of variation, ranging from 2 to 122 Gm. The average ovarian weight was greatest for the calves receiving no estrogen, but the lightest as well as heaviest ovaries were both in this group. This variation cannot be explained on the basis of information at hand, but its occurrence should be ever in mind when gonadotropic hormone treatment is being given. Quantitative results would appear to be much less predictable than qualitative results. Corpora lutea probably can be produced with greater surety than any given size of ovary in a standard length of time. The condition of the ovaries in the group autopsied forty days after the end of treatment suggests, however, that even in the case of excessive stimulation, the animal may be able to correct the condition and eventually return to a normal or near normal degree of ovarian development.

Normal Cows Injected Subcutaneously with Pituitary Gonadotropins. — Twenty-three cows which would appear to be normal in reproductive behavior, as judged by their breeding history and clinical observations, were injected subcutaneously with pituitary gonadotropins (table 2). Most of these cows were examined per rectum just before treatment was begun and stimulation of the ovaries could be determined by an increase in size from the time of examination until autopsy.

Various stages in the reproductive cycle are represented by the different cows. The postpartum cows had shown no heat period since calving, and examinations of the reproductive organs showed that quiet ovulation had not occured. Those cows classified as "post-quiet ovulation" had shown no heat period since calving, but examination of the ovaries per rectum every few days had indicated that quiet ovulation had occurred.

All classes of cows reacted to the gonadotropic treatment. This is indicated by the weights of the ovaries and in some cases by formation of new corpora lutea. Casida[14] reported the average weight on ovaries

[13]Casida, L. E., Chapman, A. B., and Rupel, I. W.: Ovarian Development in Calves. J. Agri. Research. *1*, (1935), pp. 953-960.

[14]Casida, L. E.: Bovine Ovarian Reactions to Various Gonadotropic Hormone Preparations. J. of Dairy Science. *19*, (1936), pp. 448-450.

from 20 normal, uninjected cows as 18 ± 1.2 Gm. The averages for different breeds of dairy cows reported by Swett, *et al.*[15], ranged from 19.6 to 24.7 Gm. Most of the injected cows (table 2), therefore, showed ovarian weights beyond the probable range occurring in untreated animals.

It was stated above that none of the calves treated by subcutaneous injections alone had formed corpora lutea six days after the beginning of treatment. Six of the 20 cows that were slaughtered at six days had formed new corpora lutea. Of these, cow 49 was pregnant 132 days at the beginning of treatment and cows 99 and 93 were postpartum twenty-one and seventeen days. Cows 88 and 86 were fifteen and fourteen days, respectively, after the occurrence of a quiet ovulation, and cow 90 was sixteen days after the occurrence of estrus. The data fail to indicate that the stage of the reproductive cycle affected the quantitative response to the hormone; however, they are not in such a form that a sensitive test can be applied to this question.

At the time that cow 50, the first postpartum cow, was treated, it was suspected that there would be very little reaction to gonadotropins and consequently, 12-Gm. equivalents, a rather large dose, was administered. This was suspected because previous observation had indicated that follicular development is at a very low ebb during the first two or three weeks after parturition (Casida and Venzke[16]). The response obtained from this cow, ovaries weighing more than a half pound, made it appear that our judgment had been wrong. Besides their large size, there were two other points of interest with regard to these ovaries. Although there had been excessive stimulation as far as weight is concerned, follicles were no larger than 20 mm. in diameter. The second point was that the ovarian tunics were ruptured all along the greater curvatures of both ovaries. This left the follicular walls grossly exposed. Apparently, follicular development had been so great and so rapid that the ovarian tunic could not accommodate them.

It is recognized that follicular development is at a low ebb during pregnancy, particularly during the later stages. Hammond[17] pointed out that no large follicles were present in heifers killed during the sixth and seventh month of pregnancy. He also quotes Kupfer as finding that the number of follicles in the ovary visible to the naked eye decreased toward the middle and end of pregnancy. Unpublished data at the Wisconsin Experiment Station (Casida and Chapman) have indicated that the average diameter of the largest follicles decreased from 12 mm. in the second month to approximately 9 mm. at the fifth month and to 4 mm. in the eighth or ninth month. Vesicular follicles were always present, but the degree of development was small during the later stages of pregnancy.

Two questions were in mind with regard to the treatment of the pregnant cows (47 to 49, 51, 55, 56, and 96): "Would the ovaries of pregnant cows react as readily as those of open cows?", also, "Would excessive follicular stimulation cause an abortion?"

As judged by the weights of the ovaries at the time of autopsy and also by the sizes of the largest follicles in the ovaries, there seems to be no reason for considering pregnant animals any less reactive to pituitary gonadotropins than nonpregnant animals. The 4 animals injected with (FS-1)17 and 19 can be compared, in dosage and interval from beginning of treatment to autopsy, with cows 44 and 46, cited in table 4. The ovarian weights in the first case ranged from 33 to 127 Gm., whereas in the 2 latter animals, it was 124 and 132 Gm. Another direct comparison can be made between cows 55 and 56 (table 2) and 71 (table 4). The ovary weights were 29 and 82 Gm. in the pregnant and 56 Gm. in the open cows.

None of these animals showed any evi-

[15]Swett, W. W., Matthews, C. A., Miller, F. W., and Graves, R. R.: Variations Recorded in the Study of the Conformation and Anatomy of 593 Dairy Cows Having Records of Production. U. S. Dept. Agric., BDIM-589, Revised, (1937).

[16]Casida, L. E., and Venzke, W. G.: Observations on Reproductive Processes in Dairy Cattle and Their Relation to Breeding Efficiency. Proc. Am. Soc. Anim. Prod., (1936), pp. 221-223.

[17]Hammond, John: Reproduction in the Cow. Cambridge University Press, London, (1927), 217 pp.

TABLE 2.—Normal Cows Injected Subcutaneously with Pituitary Gonadotropins.

Animal No.	Reproductive stage —days*	Subcutaneous Injections: Extract Type and No.†	Dosage —Gm. equivalents	No. of Injections	‡Days before observation	Ovarian wt. —Gm.	No. of corpora lutea	Diam. of largest follicle —mm.
50	PP10	Unf.	12	6	6	235	0	20
47	Pr64	(FS-1)17	8	4	6	33	0	17
48	Pr123	(FS-1)17	8	4	6	127	0	18
49	Pr132	(FS-1)17	8	4	6	58	0	19
51	Pr128	(FS-1)19	8	4	7	121	0	20
55	Pr134	Unf. 10CRWI	8	4	6	82	0	24
56	Pr140	Unf. 10CRWI	8	4	6	29	1	19
96	§Pr (88 cm.)	Unf. 87HMI	4	4	6	86	0	23
99	PP21	Unf. 87HMI	4	4	6	41	9	18
88	PQO15	Unf. 87HMI	4	4	6	55	1	16
97	PQO4	Unf. 87HMI	4	4	6	73	0	15
83	PE15	Unf. 87HMI	4	4	6	50	0	25
91	PE16	Unf. 87HMI	4	4	6	60	0	23
93	PP17	Hemin +	320 mg.					
		Unf. 87HMI	4	4	6	39	1	12
98	PP40	Hemin +	320 mg.					
		Unf. 87HMI	4	4	6	45	0	19
100	PPO	Hemin +	320 mg.					
		Unf. 87HMI	4	4	6	13	0	18
101	PP8	Hemin +	320 mg.					
		Unf. 87HMI	4	4	6	33	0	20
94	PP46	Hemin +	320 mg.					
		Unf. 87HMI	4	4	6	100	0	18
86	PQO14	Hemin +	320 mg.					
		Unf. 87HMI	4	4	6	64	2	15
85	PE15	Hemin +	320 mg.					
		Unf. 87HMI	4	4	6	54	0	18
90	PE16	Hemin +	320 mg.					
		Unf. 87HMI	4	4	6	30	7	8
171	PE10	Unf. S and X	105	35	35	1425	Many	50
173	PQO20	Unf. S1 and 2	120	40	41	1440	Many	55

*Pr = Pregnant; PP = Postpartum; PQO = Post-quiet ovulation; PE = Postestrus.
†For explanation of abbreviations, *see* p. 77. ‡Interval calculated from beginning of treatment. §Breeding date not known; length of fetus at autopsy given.

dence of a disturbance of her pregnancy during the six or seven days from the beginning of treatment until autopsy. Neither did any of them show any evidence of estrus, despite the great amount of follicular development in many of them. Examination of the fetus and placental attachments at the time of autopsy showed no evidence of abnormality. The period for possible disturbance in these animals, however, was relatively short. Further evidence will be cited regarding the effect of the treatment.

Five nonpregnant cows were injected with 4-Gm. equivalents of unfractionated extract 87HMI. Eight other cows belonging to the same general classification were injected with the same quantity of the same extract to which had been added, as an *in vitro* mixture, 320 mg. of heme. A comparison of the ovaries from these 2 groups of cows constitutes a test of the ability of heme to produce augmentation of ovarian weights when administered as a mixture with unfractionated pituitary extract. The average ovarian weight for the group receiving pituitary extract alone was 56 Gm., whereas that for the group receiving pituitary extract plus heme was 47 Gm. It was pointed out above, in relation to the pituitary extract and heme treatment of calves,

that there was no evidence of augmentation, and these data on cows are in agreement.

There was a significant difference, however, in the sizes of the largest follicles in the ovaries of the 2 groups of cows. For the cows receiving unfractionated extract alone (table 2), the average was 20.33 mm., and for the 8 cows receiving heme in addition to the unfractionated extract, it was 16 mm. The reason for this is not clear, but for ovaries of the same weight it might suggest that more follicles had been stimulated but to a lesser degree when heme was added to the extract. The average size of the largest follicles for the 4 cows receiving (FS-1) was 18.75 mm., which also tended to differ from the average on the unfractionated extract.

The treatments for the longest durations to which any of the cows were subjected were those of 171 and 173. These received 35 and 40 injections, respectively. They were injected with 3-Gm. equivalents of unfractionated extract daily for long periods of time, to determine the readiness with which cattle develop antigonadotropic hormone. Tests of 2 cc. of the blood serum from these cows against pituitary extract in immature rats showed no evidence of antihormone being present until approximately twenty-two days after the beginning of treatment in cow 171. The inhibitory action was slight at that time, but even such a slight effect did not show up in 173 until between thirty-six and thirty-nine days after treatment began. The most extreme effect was in 171 when, at thirty and thirty-five days, 2 cc. of serum inhibited the response of test rat ovaries to 27 mg., whereas the control ovaries weighed 65 mg. None of the responses was judged to be more than slight.

Unusual reactions to the long treatment were encountered in these cows. The first cow to be treated (171) went off feed twenty-four days after the beginning of treatment but, despite her lack of appetite, she showed an increasing fullness of the abdomen. Examination of the animal per rectum indicated that there was an accumulation of fluid within the abdominal cavity. A clear, yellowish, and odorless fluid was obtained by tapping the abdomen in the ventral region. The cow was slaughtered thirty-five days after the beginning of treatment, and approximately 20 gal. of fluid escaped from the abdomen when it was opened.

Cow 173 did not go off feed until thirty-seven days after the beginning of treatment and, at that time, fluid could be palpated in the abdominal region per rectum. The cow was tapped and clear fluid could be drained from the abdomen. Between thirty-seven and thirty-nine days after treatment began, the cow changed distinctly in appearance from bloated to very gaunt. During this time, the herdsman reported that the cow was urinating excessively, both in frequency and amount. Tapping of the abdomen after this period failed to yield any fluid. The cow was weighed 4 times during the course of treatment. At five days, she weighed 1,110 pounds, twenty-nine days—1,177, thirty-six days—1,230, forty days—1,025. The change in body weights between the thirty-sixth and fortieth days is probably also indicative of loss of accumulated fluid. No excessive amount of abdominal fluid was present at autopsy, forty-one days after the first injection.

The experiment itself furnishes no facts for explaining the development of this condition of ascites. The observation that the fluid coagulated upon exposure to the air suggests that it was a transudate, but this also is a condition sometimes observed in follicular fluid. Injection of unfractionated sheep pituitary extract into a ewe for forty-three days (unpublished data, Wisconsin Agricultural Experiment Station) resulted in loss of appetite and death at the end of that time. More than a gallon of fluid was recovered from the abdomen at autopsy. Other work failed to produce this condition in rabbits by longtime injections, but antihormones are easily produced in rabbits. The phenomenon seems to be associated with persistent treatment of animals in which antihormones are not readily developed.

The largest ovaries obtained from any cows were found in these 2 at the time they

were slaughtered. Those of 171 weighed 1,425 Gm. and those of 173, 1,440 Gm. Strikingly enough, these weights are approximately 60 times the normal, as was observed for the largest calf ovaries. The responses represented by these ovaries might well serve as a warning of the harmful results of excessive treatment. The ovaries contained large numbers of corpora lutea, but in general there was not complete luteinization. Many cysts and partially luteninized cysts up to 50 and 55 mm. in diameter were present.

Fig. 2.—Genital Organs from Cow 171.
This cow had been injected for thirty-five days and at the autopsy, the ovaries weighed 1,425 Gm.

Normal Cows Treated by Subcutaneous Followed by Intravenous Injection of Pituitary Gonadotropins.—The cows treated by this method are listed in 5 groups (table 3) to facilitate discussion. Those of group 1 were pregnant when treatment began. Cow 131, treated with an unfractionated extract, showed the formation of 15 corpora lutea when she was slaughtered thirty-four days later. Cow 146, treated with a purified follicle-stimulating extract, showed the formation of no additional corpora lutea, and any follicular developments that may have occurred had regressed by the time of slaughter twenty days later. Neither of these cows showed any evidence of estrus at any time following treatment, and, what is more important, the pregnancies at no time appeared to be endangered, and no harmful effects were evidencd on the fetus or attachment of fetal membranes at the time of autopsy.

The cows in group 2 were all treated with essentially the same dosage of the same unfractionated extracts. They were, however, slaughtered at different intervals following the initiation of treatment. Some of these animals were bred and some were not. Most of these cows did not come into heat near the time of the intravenous injection. In this case, the cows were usually "force bred" to an aggressive young bull the day of the intravenous injection and again on the next day.

Probably, in no case was the onset of the next estrus accelerated by treatments with the gonadotropins, with the exception of cow 108, which received, in addition, 20,000 rat units of Progynon B. In her case, the estrus observed seven days after the beginning of treatment is attributable to the estrogen and not to the gonadotropin. It would appear that although excessive numbers of follicles are stimulated by an unfractionated extract, these follicles are incapable of inducing estrus, or the process is interfered with by the formation of corpora lutea.

Cow 109 was in heat five days after the beginning of treatment, but this was also twenty days after her last natural heat period, so that it might be considered as associated with her natural estrual cycle. Aside from the heat period shown in connection with the injection of Progynon B, 108 showed no heat up to the time she was slaughtered, which was forty days following her preceding natural heat period. The fact that the ovary showed a recent ovulation indicates that heat was not expressed following the treatment despite the fact that these cows were being checked with a bull twice daily. Two of the cows (114 and 121) were slaughtered fifteen and seventeen days following their last natural heat period, so there was no opportunity to detect the effect of treatment upon the natural estrual cycle. Cow 115 was in heat thirty-one days after her last natural estrus, but it might be reasoned that inasmuch as she was bred, a pregnancy was initiated but not

TABLE 3.—Normal Cows Treated by Subcutaneous Followed by Intravenous Injections of Pituitary Gonadotropins.

			Subcutaneous Injection			Intravenous Injection							
Group No.	Animal No.	Reproductive stage —days*	Extract Type and No.†	Dosage —Gm. equivalents	No. of Injections	Extract Type and No.†	Dosage —Gm. equivalents	‡Days before observation	Ovarian wt. —Gm.	No. of corpora lutea	Diam. of largest follicle —mm.	‡Days before estrus	Results of Insemination
1	131	Pr84	Unf.87HMI	4	4	Unf.87HMI	1	34	37	15	..	..	
	146	Pr57	(FS-2)105WS	9	3	(FS-2)105WS	3	20	19	0	8	..	
2	111	PE2	Unf.87HMI	4	4	Unf.87HMI	1	32	46	25	19	..	Not pregnant at autopsy
	115	PE2	Unf.87HMI	4	4	Unf.87HMI	1	31	28	39	4	29	Not pregnant at autopsy
	114	PE4	Unf.87HMI	4	4	Unf.87HMI	1	11	28	0	20	..	
	108	PE4	Unf.87HMI	4	4	Unf.87HMI	1	36	27	3	17	7§	Not pregnant at autopsy
	116	PE5	Unf.87HMI	4	4	Unf.87HMI	1	31	174	3	28	..	Not pregnant at autopsy
	121	PE6	Unf.87HMI	4	4	Unf.87HMI	1	11	48	13	23	..	
	110	PE7	Unf.87HMI	4	4	Unf.87HMI	1	17	20	8	..	..	
	107	PE12	Unf.87HMI	3.4	4	Unf.87HMI	1	17	80	26	36	..	
	112	PE12	Unf.87HMI	4	4	Unf.87HMI	1	15	81	37	25	12	
	106	PE15	Unf.87HMI	4	4	Unf.87HMI	1	16	43	24	22	..	Not pregnant at autopsy
	109	PE15	Unf.87HMI	4	4	Unf.87HMI	1	12	30	7	15	5	1 fragmenting ovum.
3	153	PE2	(FS-2)100CS	9	3	(FS-2)100CS	3	7	89	1	25	..	
	150	PE3	(FS-2)104CS	6	2								
			(FS-2)107	3	1	(FS-2)107	3	8	30	7	18	..	
	147	PE5	(FS-2)102WS	9	3	(FS-2)102WS	3	9	25	2	18	6	
	151	PE5	(FS-2)100CS	9	3	(FS-2)100CS	3	10	61	0	24	..	
	145	PE9	(FS-2)100WS	9	3	(FS-2)100WS	3	6	33	1	19	5	
	154	PE9	(FS-2)100CS	4.5	3	(FS-2)100CS	1.5	9	66	24	18	..	
	149	PE9	(FS-2)100CS	9	3	(FS-2)100CS	3	10	58	15	25	4	
	155	PE9	(FS-2)100CS	4.5	3	(FS-2)100CS	1.5	10	24	5	12	..	
	160	PE9	(FS-2)110WS	4.5	3	(FS-2)110WS	1.5	9	14	0	17	9	
4	176	PE2	(FS-2)240WS	9	3	(Lut-2)13HM2	5	..	..	6‖	..	15	Resumed estrual cycle
	179	PE2	(FS-2)240WS	9	3	(Lut-2)13HM2	7	..	..	4‖	..	19	Resumed estrual cycle
	183	PE2	(FS-2)240WS	9	3	(Lut-2)13HM2	5	8	36	4	10	..	4 1-cell eggs
	185	PE2	(FS-2)240WS	9	3	(Lut-2)13HM2	5	..	..	5‖	..	27	Resumed estrual cycle
	184	PE3	(FS-2)240WS	9	3	(Lut-2)13HM2	5	7	36	0	19	..	Did not ovulate
5	180	PE16	(FS-2)15HM2	16.2	3	(Lut-2)18HM2	7	36	29	9	13	..	7 embryos, 10-13 mm.
	174	PE17	(FS-2)240WS	9	3	(Lut-2)13HM2	7	50	20	6	11	..	4 embryos, 3-32 mm.
	175	PE17	(FS-2)403WS	16.2	3	(Lut-2)18HM2	7	..	..	4‖	..	..	Pregnant at 43 and 51 days, not at 70 days
	177	PE17	(FS-2)403WS	16.2	3	(Lut-2)18HM2	7	33	36	5	5	4	4 embryos, 9-11 mm.
	178	PE17	(FS-2)240WS	9	3	(Lut-2)13HM2	7	..	..	1‖	..	17	Resumed estrual cycle
	181	PE17	(FS-2)240WS	9	3	(Lut-2)13HM2	7	..	..	0‖	..	3	Resumed estrual cycle
	**176	PE18	(FS-2)403WS	16.2	3	(Lut-2)18HM2	7	..	..	2‖	..	25	Resumed estrual cycle

*Pr = Pregnant; PE = Postestrus. †**For explanation of abbreviations**, *see* p. 77. ‡Interval calculated from beginning of treatment. §20,000 rat units of Progynon B injected subcutaneously, **four days after beginning** of treatment. ‖Number determined by rectal palpation of ovaries at fifty-one days. **Cow was treated four months previously in PE2 stage.

maintained. Cow 112 was not bred, but estrus was delayed until twenty-four days after the last natural heat. Three other cows that were bred (111, 116, and 106) had gone thirty-four, thirty-six, and thirty-one days, respectively, before slaughter, but no estrus had been expressed in the meantime. None of these showed any evidence of pregnancy at the time of slaughter. Three other cows that were not bred (110, 107, and 112) had gone twenty-four, twenty-nine, and twenty-seven days, respectively, since their last heat period at the time of slaughter and also had not shown the occurrence of estrus. From these data, it would appear that treatment with the unfractionated extract does not initiate estrus itself and also that the occurrence of the next heat period is delayed, *i.e.*, the timing of the natural estrual cycle is disturbed. It might be reasoned that the failure of pregnancy to result from treatment with unfractionated extract is due to the absence of behavioral estrus or something associated with it. Cow 108 was injected with Progynon B to test this idea. Estrus was induced, but still no pregnancy was evidenced thirty-six days after the beginning of treatment. It might appear from the ovum recovered from cow 109 that failure was occurring in the very early stages, either a failure of fertilization or the degeneration of the embryo while in the very-few-cell stage.

The cows in group 3, treated entirely with purified follicle-stimulating extracts, shed some light on the production of ovulation and estrus during the natural interestrual period. Four of these cows (147, 145, 149, and 160) showed estrual behavior six, five, four, and nine days, respectively, after the beginning of treatment. These, in turn, were eleven, fourteen, thirteen, and eighteen days after the last natural heat period and would appear to be some acceleration over the natural estrual rhythm.

Seven of these 9 cows showed the presence of young corpora lutea at the time of autopsy. Inasmuch as all of these cows were slaughtered before the time of an expected natural heat period, it would appear that the follicle-stimulating extract, when injected subcutaneously and then intravenously, can initiate the formation of corpora lutea without the treatment being superimposed upon whatever natural forces produce ovulation in connection with the natural estrual period. The initiation of corpora lutea formation, only, has been mentioned. From the appearance of rupture points, it is assumed that most of them were corpora lutea of ovulation; no actual search for eggs was made. It also is not known whether these corpora would continue their development without some further treatment or the intervention of the cows' own endocrine activity. As was pointed out above, where calves were treated with the purified follicle-stimulating extracts, it seems best to assume that the cow's own pituitary gland may have played a part in the production of ovulation. It would be desirable to run another series of animals treated by the subcutaneous method only, to see if ovulation would be produced during the interestrual phase; the effect of the intravenous injection cannot be evaluated from this experiment. The fact that estrus occurred in a part of these cows during the interestrual phase and did not in the ones treated with unfractionated extract leads one to believe that the fractionation process removes something from the extract which through its action, probably on the ovaries, inhibits the estrus normally expected from follicular maturation.

The cows in group 4 were injected subcutaneously with a purified follicle-stimulating extract followed by an intravenous injection of a purified luteinizing preparation. All of these cows were in the early stages of the estrual cycle, being two to three days past the natural period when treatment began. The cows were artifically inseminated on the day of the intravenous injection and usually on one or two days following this time. One of the animals (184) did not ovulate and the other 4 animals, in which there was reason to believe ovulation had occurred, did not become pregnant.

Cow 183 was killed eight days after treatment was begun, to see if the eggs were fertilized. Four 1-celled eggs were recovered from the oviduct; fertilization

had not taken place. Cows 176, 179, and 185, were in heat seventeen, twenty-one, and twenty-nine days, respectively, after their last natural heat periods. None of them showed estrus in connection with the treatment during the early part of the estrual cycle. Little can be said as to the effect of treatment upon natural estrual rhythm, cow 176 coming back a little early and 185 being delayed, whereas 179 was back in heat on time.

In contrast to the cows in group 4, which were injected during the early or luteal phase of the estrual cycle, the cows in group 5 were injected first from the sixteenth to the eighteenth day, or during the follicular phase of the cycle. The dosage and injection method, as well as extracts used, were essentially the same as those used on the cows in group 4. Artificial insemination was carried out in much the same way, being timed in relation to the intravenous injection. Objective evidence of the occurrence of pregnancy was obtained in 3 of these cows. Cow 180, which was slaughtered thirty-six days after the beginning of treatment, showed the presence of 7 embryos ranging from 10 to 13 mm. in length. One of these showed considerable hemorrhage into the amniotic fluid, and this may have been an indication of embryonic death either having occurred or being imminent. There were 9 corpora lutea with papillae present, ranging in size from 12 to 17 mm. in diameter. From cow 174, which showed 6 corpora lutea ranging from 13 to

Fig. 3.—The Seventh Embryos from Cow 180. There has been hemorrhage into the amniotic vesicle surrounding one of these.

Fig. 4.—One Ovary and the Three Normal Embryos from Cow 174. A fourth embryo, which was degenerating, is not clearly visible in the photograph.

17 mm. in diameter at the time of her autopsy fifty days after the beginning of treatment, 3 normal-appearing embryos ranging from 26 to 32 mm. in length were recovered. There was also 1 small degenerating embryo 3 mm. in length present. There were 5 corpora lutea in cow 177, the third cow which showed definite evidence of pregnancy, but only 4 of these corpora lutea had papillae present. This cow was killed thirty-three days after the beginning of treatment, and she showed the presence of 4 embryos ranging from 9 to 11 mm. in length at the time of autopsy.

A fourth cow (175) should be classed as pregnant, but the data on her are on an entirely different basis. Definite pregnancy diagnoses were made forty-three and fifty-one days after the time of the intravenous injection. These diagnoses were based on the increased size of the uterine horns and upon the presence of fluctuating fluid contents within the horns as determined by rectal palpation. The third examination was made at seventy days. The horns had returned to normal size and there were obviously no contents, as detected by palpation. This cow was killed on the next day, and was found not to be pregnant. A recent quiet ovulation had occurred from which an unfertilized ovum was recovered from the oviduct by flushing with physiological saline. Any previous corpora lutea that may have been present had apparently degenerated beyond recognition at autopsy; 4 corpora lutea were palpable at fifty-one days.

The other 3 cows in group 5 resumed their estrual cycles, cow 178 returning in heat after seventeen days and 176 after twenty-five days; 181 resumed her cycle thirty-two days after the heat period which was expressed in connection with the treatment. The latter was 1 of the 2 cows which showed estrus in connection with the treatment with follicle-stimulating extract, cow 177 being the other. Of the 4 cows, therefore, that appeared to have been pregnant, only 1 of them showed a heat period at the time of insemination. The cows that resumed their estrual cycle after treatment and breeding differed from the cows that conceived, in the results observed by manual palpation of the ovaries. Cow 181 showed the presence of no corpora lutea, and 178 and 176 showed 1 and 2 corpora lutea, respectively. The numbers present in the pregnant cows ranged from 4 to 9. Cow 181, of course, could not be expected to conceive because of the failure of ovulation. It is not known whether 178 and 176 failed in part because of the low ovulation rate in connection with an excessive stimulation of follicular development.

The cows in group 5 demonstrate the potential fertility of eggs from follicles artificially matured by gonadotropic hormone. The ovulation stimulus may, however, have been enhanced by changes occurring in the animal in connection with her own estrual rhythm. An indication is given by this group of cows that estrual behavior is not necessary for the production of fertility.

Nothing can be told with certainty regarding the pregnancies that were established in the slaughtered cows, as to whether they would have gone ahead to produce multiple-births. There was evidence that 2 of the embryos were regressing at these stages. The fact that the pregnancy in another cow (175) regressed between fifty-one and seventy days also questions the ability of these pregnancies to be maintained.

A comparison of the breeding results from groups 4 and 5 makes it appear that there is a difference in the potential fertility of eggs matured during the 2 different stages of the estrual cycle. From the 1 luteal-phase cow (183), which was autopsied at a stage in which fertilization of the eggs could be determined, the failure of fertility in these cases would appear to be due to failure of fertilization. There are reasons to believe that the sperm in the 2 groups of animals were comparable in their potential fertility. This apparent difference in fertility resulting from animals treated in the luteal and follicular phases of the cycle needs further study.

Cows with Incomplete or Questionable Breeding Histories.—As pointed out earlier, these animals were usually being discarded for reasons other than extremely

low breeding efficiency. They showed no gross abnormality of the reproductive organs at the time of clinical examination. It seems justifiable to include these animals because of the difficulty in obtaining sufficient numbers for study of so-called normal animals and, what is perhaps more important, the responses of these animals seemed in all essential respects to corroborate the findings on normal animals.

An attempt was made with cow 40 (table 4) to induce a nymphomaniac condition, using an (FS-1) extract. Behavioral estrus did not occur and corpora lutea were present in the ovaries at the time of slaughter.

Cows 44 and 46 have already been discussed in connection with the responses of cows 47 and 48 (table 2). Again, cows 57 and 71 on unfractionated extracts produced follicles up to 20 and 23 mm. in diameter, as compared with 16 and 17 mm. diameter for cows 44 and 46 on (FS-1) extract. This difference was true whether or not corpora lutea had been formed.

Four cows (42, 43, 61, and 62) were animals that had been bred 4 to 7 times without conceiving. These cows were treated beginning from the fourteenth to the sixteenth day of their estrual cycles, (FS-1) extract being used on the first 2 and unfractionated extract on the last 2. They were bred in connection with the accompanying heat periods and the 2 animals on the follicle-stimulating extract became pregnant. One of the animals on the unfractionated preparation did not ovulate and the other did not develop a normal pregnancy. When she was slaughtered at twenty-three days, 9 eggs were recovered from the uterine horns. One of these eggs appeared to have reached a 3-cell stage and 4 others the 2-cell stage before fragmentation began. This description implies that fertilization of a part of the eggs occurred and, of course, is an interpretive one. This last animal did indicate that when corpora lutea were formed as a result of administering unfractionated extract during the follicular phase of the cycle, some ovulation at least might occur, but the ability of the eggs to be fertilized or to develop normally if fertilized was decidedly in question. This seemed to be in contrast to the cows that were treated with follicle-stimulating extract during the follicular phase of the cycle.

Cows 72 and 76 furnished information that unfractionated extracts when injected first subcutaneously and then intravenously would produce ovulation, as indicated by the recovery of 5 eggs from the oviduct of 72 and 3 from 76. The question had been raised up to this time as to whether the eggs might not all be entrapped in the corpora lutea formed by such treatment.

An attempt was made with cow 135 to maintain the experimentally produced corpora lutea by means of continued subcutaneous injection of the unfractionated extract, and if possible, to inhibit the natural estrual rhythm. The animal was in heat nineteen days after the beginning of treatment, and at the time of autopsy there was evidence that the experimenally produced corpora lutea had regressed and new corpora lutea were formed in connection with the heat period.

Trials were made with cows 75, 132, 133, and 134 to initiate pregnancy from ovulations induced by unfractionated extract. Three of these animals were in the luteal phase of the estrual cycle. The only ones of these animals that showed estrus in connection with treatment were the ones that received Progynon B in addition to the gonadotropin (see table 4). These last 3 animals were allowed to go until thirty-one to thirty-five days after the beginning of treatment when they were slaughtered. No evidence of pregnancy was obtainable at that time. Prior to slaughter, however, these cows had come into estrus at intervals of twenty-three to twenty-eight days following the beginning of treatment (estrual cycles of twenty-eight to thirty-two days). This is in agreement with the delay of the succeeding estrus following treatment which was noted in connection with normal cows treated by subcutaneous injections of unfractionated extract. Treatment with unfractionated pituitary extract plus estrogen during the luteal phase of the estrual cycle failed to produce pregnancy in these cows.

TABLE 4.—Cows with Incomplete or Questionable Breeding History, but Showing no Abnormality at Clinical Examination.

Animal No.	Reproductive stage—days*	Extract Type and No.†	Dosage—Gm. equivalents	No. of subcutaneous injections	No. intravenous injections	‡Days before observations	Ovarian wt.—Gm.	No. of corpora lutea	Diam. of largest follicle—mm.	‡Days before estrus	Results of Breeding
40	Unknown	(FS = 1)10	66	22	0	23	54	8	10	..	
44	PE4	(FS-1)17	8	4	0	6	124	0	17	..	
46	PE1	(FS-1)17	8	4	0	6	132	7	16	..	
57	Unknown	Unf.10CRWI	8	4	0	6	66	0	20	..	
71	PE2	Unf.10CRWI	4	4	0	6	56	0	23	..	
42	PE15	(FS-1)17	8	4	0	..	..	6§	..	4	Live calf at 265 days
43	PE16	(FS-1)17	8	4	0	58	16	1	15	5	32-mm. embryo
61	PE14	Unf.10CRWI	8	4	0	17	70	0	36‖	7	Did not ovulate
62	PE15	Unf.10CRWI	8	4	0	23	100	25	30	8	4 1-cell, 4 2-cell, 1 3-cell fragmenting eggs, 2 1-cell ova from oviduct.
72	Unknown	Unf.10CRWI	8	3	1	6	33	8	..	..	
76	Unknown	Unf.54LECI	6 2/3	3	1	6	36	14	..	..	
135	PE2	Unf.87HMI	5	4**	1	27	14	12††	15	19	
75	Unknown	Unf.10CRWI	8	7	1	33	18	4	16	..	Not pregnant at autopsy
132	PE4	Unf.87HMI	6	5	1	35	21	24	15	6‡‡	Not pregnant at autopsy
133	PE5	Unf.87HMI	5	4	1	31	56	34	..	3§§	Not pregnant at autopsy
134	PE5	Unf.87HMI	5	4	1	31	18	9	20	3§§	Not pregnant at autopsy

*PE = Postestrus. †For explanation of abbreviations, *see* p. 77. ‡Interval calculated from beginning of treatment. §Number determined by palpation forty-nine days after heat. ‖Partially luteinized. **Also 18 subcutaneous injections of 0.25-Gm. equivalents following intravenous injection. ††Also 2 corpora lutea from heat nineteen days after beginning of treatment. ‡‡20,000 rat units of Progynon B, given subcutaneously three days after beginning of treatment. §§10,000 rat units of Progynon B, given subcutaneously three days after beginning of treatment.

SUMMARY

When unfractionated pituitary extracts were administered subcutaneously to calves, the formation of corpora lutea seemed to be associated more closely with the interval of time during which the hormone was acting than it did with the size of the ovary produced or with the production of a certain minimum size of follicle.

A calf with no vesicular follicles in the ovaries failed to respond to gonadotropic treatment. Another calf in which the follicles had been destroyed by electrical cautery did respond, however. The ovarian response to a gonadatropic treatment is probably not affected so much by the number of follicles visible in the ovary *per se* as it is by some basic condition which has produced or maintained those follicles in the first place.

Heme failed to increase the quantitative response to unfractionated pituitary extracts (ovarian weight) when administered to calves or to cows, but the diameter of the largest follicles averaged smaller in those cows that received both heme and pituitary extract than in those that received the extract alone. This was not observed in calves.

Unfractionated extracts from beef pituitary glands gave quantitative responses in calves within the range observed from treatment with unfractionated extracts of sheep pituitary glands.

Ovulation could be induced in calves within a shorter time when treated by subcutaneous injections of follicle-stimulating extract which were followed by an intravenous injection of a luteinizing extract, than when treated by subcutaneous injections of either follicle-stimulating or an unfractionated extract alone.

The corpora lutea formed as a result of gonadotropic treatment in calves appeared to persist as morphological structures for as much as twenty-three days but disappeared by forty days after the beginning of treatment.

Eggs from artificially matured and ovulated follicles of calves were fertilizable but the fertilization rate was extremely low. Out of 19 eggs recovered from calves that had been inseminated, only 2 were fertilized and a third was questionable.

The maximum response to gonadotropic extract in calves and cows appeared to be an increase of approximately sixty-fold in ovarian weight. Quantitative responses would appear to be much less predictable than qualitative results. Corpora lutea were produced with more surety than any limited range of ovarian size in a standard length of time.

No differences in the weights of ovaries produced by similar treatments with pituitary gonadotropins were detected in cows representing different stages of the reproductive cycle. These stages included early, middle, and late pregnancy, postpartum, post-quiet ovulation, and the follicular and luteal phases of the estrual cycle.

Neither excessive follicle stimulation nor the formation of additional corpora lutea gave evidence of producing abortion in pregnant cows up to thirty-four days after the beginning of treatment.

Injection of unfractionated sheep pituitary extracts into cows daily for five to six weeks caused the development of excessive amounts of abdominal fluids and impairment of the animal's health.

The injection of unfractionated extracts during the early or luteal phase of the estrual cycle did not produce heat. The next anticipated estrus of the natural cycle was delayed as a result of this treatment. In some, but not all, cases there seemed to be interference with the expression of the natural estrus when injections were made during the latter part of the estrual cycle.

Estrus appeared to be neither hastened nor delayed when cows were treated during the early part of the estrual cycle by subcutaneous injections of a follicle-stimulating extract followed by an intravenous injection of a luteinizing extract. This same treatment during the latter part of the estrual cycle in some cases appeared to interfere with the expression of natural estrus.

The only treatment which appeared to accelerate the appearance of estrus was with subcutaneous followed by intravenous

injections of a purified follicle-stimulating extract.

Cows treated subcutaneously for four days with unfractionated extracts produced larger follicles than cows similarly treated with follicle-stimulating extracts. This seemed to be true whether corpora lutea resulted from the treatment or not.

Ovulation resulted fairly consistently in cows from subcutaneous followed by intravenous injections either when both types of injection were of an unfractionated extract or when the subcutaneous injections were follicle-stimulating in character and the intravenous injections were luteinizing. Corpora lutea also were formed in 7 of 9 cows that were injected both subcutaneously and intravenously with follicle-stimulating extracts during the luteal phase of the estrual cycle.

Fertility did not result from insemination of cows treated either with unfractionated extracts or with follicle-stimulating followed by luteinizing extracts during the early part of the estrual cycle. Likewise, evidence of only slight fertility followed by early embryonic death was obtained from cows treated during the latter part of the estrual cycle with unfractionated extracts. Fertility was obtained during the latter part of the estrual cycle when cows were treated with follicle-stimulating extracts or by follicle-stimulating followed by luteinizing extracts. Even then, there was some evidence of early fetal death in cases of hyperfecundation.

The Disinfection of Baby Chick Shipping Boxes with Sodium Silicate Solutions

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AN ACUTE SHORTAGE of the cardboard boxes used for shipping baby chicks now exists and probably will continue for the duration of the war. These boxes are being returned from the farms to commercial hatcheries for repeated use. The sanitary hazard of this practice is great because these boxes frequently are exposed to possible contamination by the adult poultry on the farms, as well as by the chicks shipped in them.

As a means of reducing this hazard, a practical method of disinfecting these boxes was sought, although it is fully recognized that no method of disinfection that must be carried out at the hatchery, and often by inexperienced operators, will make the practice of reusing baby chick shipping boxes entirely safe. To be of practical value, the treatment of the boxes has to be:

1) Effective against the common poultry pathogens, even in the presence of organic matter.
2) Stable and effective over a wide range of conditions.
3) Nontoxic to baby chicks subsequently shipped in the box.
4) Noninjurious to the box.
5) Economical, and use only materials that are readily available during the present emergency.
6) Safely and quickly applied by inexperienced labor, without the use of elaborate or expensive equipment.

Many recognized germicides were investigated. The very strong alkalies, the rapid-acting hypochlorites, and the saponified cresols were effective and economical, but had a very deleterious effect upon the box. Preliminary work with common "water glass" gave encouraging results. In addition to this commercial water glass, which is referred to in the table as "C" brand, and which is nearly a saturated aqueous solution of sodium silicate of the approximate composition $Na_2Si_4O_9$ with a small amount of the more alkaline sodium silicates and has a $Na_2O : SiO_2$ ratio of approximately 1 : 3, the following sodium silicate solutions also were investigated:

Brand*	Na_2O %	SiO_2 %	$Na_2O:SiO_2$ Ratio
"N"	8.9	28.3	1:3.2
"K"	11.0	31.9	1:2.9
"U"	13.8	33.1	1:2.4
"D"	14.5	29.0	1:2.0

The pH determinations, table 1, showed these solutions to be moderately strong alkalies, and to be sufficiently well buffered so that moderate dilution and the addition of chicken feces had comparatively little influence upon the pH values. Because of the very high sodium ion error in making electrometric pH determinations of concentrated sodium silicate solutions, the values given in table 1 should be considered as only approximate.

Previous work has shown strong alkalies to be very effective against gram-negative bacteria[1, 2, 3] and filtrable viruses,[4, 5, 6] moderately effective against gram-positive

Published as Scientific Paper No. 548, College of Agriculture and Agricultural Experiment Station, State College of Washington.

From the Division of Veterinary Science, State College of Washington.

*These products were furnished by the Philadelphia Quartz Company.

[1]McCulloch, Ernest C.: The Germicidal Efficiency of Sodium Hydroxide. J. Bact., xxv (1933), p. 469.

[2]Myers, Robert Page: The Effect of Hydroxyl-ion Concentration on the Thermal Death-Rate of *Bacterium coli*. J. Bact. clvi (1928), p. 341.

[3]McCulloch, Ernest C., and Costigan, Stella: A Comparison of the Efficiency of Phenol, Liquor Cresolis, Formaldehyde, Sodium Hypochlorite and Sodium Hydroxide against *Eberthella typhi* at Various Temperatures. J. Infect. Dis., lix (1936), pp. 281-284.

[4]Olitsky, P. K., Traum, J., and Schoening, H. W.: Report of the Foot-and-Mouth Disease Commission of the United States Department of Agriculture. U. S. Dept. Agric., Tech. Bul. 76, (1928), p. 57.

[5]McBryde, C. N., Niles, W. B., and Cole, C. C.: Experiments to Determine the Effects of Sodium Hydroxide and Calcium Hydroxide on the Virus of Hog Cholera. J.A.V.M.A., xxii (1931), p. 87.

[6]McCulloch, Ernest C.: Disinfection and Sterilization. Lea & Febiger, Philadelphia, (1936), p. 275.

nonsporulating organisms,[7] less effective against bacterial spores,[8] and relatively ineffective against acid-fast organisms.[8] Furthermore, the degree of germicidal efficiency of strong alkali solutions has been shown to be correlated with, if not directly proportional to, the degree of alkalinity acting upon the organisms.[1] Previous work[1] also has shown pH 11.5 to be sufficient to destroy *Brucella abortus* in the presence of broth in ten minutes at 40 C. The same concentration of alkali (NaOH) killed in approximately the same time at lower temperatures, and this organism is only slightly more susceptible to alkalies than is *Salmonella pullorum*. Myers[2] reported that the hydroxyl ion concentration at pH 11.1 was sufficient to kill *Escherichia coli* in ten minutes at temperatures between 25 and 40 C. He also found that exposure to pH 10 at 25 C. decreased a population of 1,510,000 per cc. of this organism to 25,000 per cc. in ten minutes. Tilley and Schaffer[8] found dilutions of 1 : 1200 of sodium hydroxide killed *S. pullorum, Shigella gallinarum* and *Pasteurella avicida* in less than ten minutes at 25 C.

The germicidal efficiencies of sodium silicate solutions were checked in three different ways: *(a)* by the standard method described by the Food and Drug Administration for the evaluation of disinfectants,[9] using *S. pullorum, E. coli* recently isolated from chick omphalitis,* *E. coli* in fresh chicken feces, and a recently isolated strain of *Staphylococcus aureus* as test organisms; *(b)* in the presence of substantial amounts of chicken feces; *(c)* under conditions paralleling the actual disinfection of chick shipping boxes, including tests of the ability to disinfect the upper layer of paper of these boxes.

Tests made by F.D.A. technic[9] show that in the absence of added organic matter, a 10 per cent solution of water glass kills *S. pullorum* in less than five minutes, and a 5 per cent solution gives variable results in ten minutes. Under parallel conditions, a 1 per cent solution of phenol usually fails to kill in five minutes, but kills in ten minutes. These data, which are presented in table 2, indicate that a 10 per cent sodium silicate solution is more effective and a 5 per cent solution is slightly less effective than is a 1 per cent phenol solution. These data indicate the phenol coefficient of sodium silicate solutions to be between 0.1 and 0.2.

TABLE 1.—Approximate pH Values of Sodium Silicate Solutions Without and with Added Chicken Feces.

Concentration	Without Added Feces				
	Commercial Water Glass				
%	"C"	"N"	"K"	"U"	"D"
100	11.9	11.6	11.9	12.2	12.3
50	11.7	11.5	11.8	12.0	12.4
30	11.6	11.4	11.6	11.8	12.2
10	11.0	11.15	11.35	11.6	11.9
	With 10% Chicken Feces Added				
100	11.5	...	11.4	...	11.7
50	11.4	...	11.5	...	12.0
30	11.3	...	11.4	...	11.8
10	11.0	...	11.1	...	11.55

These values have been approximately corrected for sodium ion error.[10] The nature of sodium silicate solutions makes exact pH measurement difficult.

Because the practicability of sodium silicate disinfection of chick shipping boxes depends upon the destruction of pathogens in the presence of fecal material, tests were made after the culture had been mixed with suspensions of chicken feces. The feces-culture mixtures were made by adding sufficient tap water to 25 Gm. fresh chicken feces to make 100 cc. This was autoclaved and when cool, 25 cc. of a 24-hour broth culture of *S. pullorum* was added. The data presented in table 3 show that when this suspension was added to an equal amount of 20 per cent sodium silicate, *S. pullorum* is killed in five, but not in two minutes. When 1 part of this suspension was added to 3 parts of 6.6 per cent sodium silicate, *S. pullorum* survived two hours, but was killed by twenty-four hours exposure.

Further tests were made using *E. coli* as a test organism because extensive research

*This culture was furnished by Dr. J. E. Lindenmayer of the Western Washington Experiment Station.

[7]McCulloch, Ernest C., and Fuller, Stewart A.: The Relative Efficiencies of Disinfectants in Killing *Erysipelothrix rhusiopathiae*. Am. J. Vet. Res., ii (1941), pp. 77-83.

[8]Tilley, F. N. W., and Schaffer, J. M.: Germicidal Efficiency of Sodium Hydroxide, Sodium Carbonate and Trisodium Phosphate. J. Agric. Res., xlii (1931), 93.

[9]Ruehle, G. L. A., and Brewer, C. M.: United States Food and Drug Administration Methods of Testing Antiseptics and Disinfectants. U. S. Dept. Agric., Circ. 198, (1931).

has established this to be one of the more resistant gram-negative organisms. It occurs in tremendous numbers in chicken feces, thus giving a natural source of infected material, and reliable techniques have been worked out for the isolation and identification of this organism from contaminated material. Comparisons of tables 2, 3, and 4 show *E. coli* is at least as resistant to sodium silicate as is *S. pullorum*.

Twenty per cent solutions of sodium silicate were poured over one-fourth their bulk of fresh chicken feces in a petri dish and allowed to stand at room temperature. Subcultures were made into lactose broth at intervals of five minutes to forty-eight hours. Even exposure to the sodium silicate for forty-eight hours failed to kill the spores of gram-positive bacilli; however, subcultures into brilliant green lactose bile broth showed that *E. coli* did not survive longer than thirty minutes.

Staph. aureus also was used in a series of tests because this organism is known to be more resistant to alkalies than any poultry pathogen except *Mycobacterium avium*. The data presented in table 5 show that only brand "D," the most highly alkaline sodium silicate, killed *Staph. aureus* in less than two hours in a 25 per cent solution, although the less alkaline commercial water glass "C," and the sodium silicate "K" killed in 24 hours. Plate count technique indicated that pure "K" brand sodium silicate, at 20 C. reduced an original *Staph. aureus* population of 530,000,000 per cc. to approximately 6,000,000 in ten minutes. Instead of continuing this rate of kill, which approached 99 per cent during each ten minute interval and which would have left approximately 1 survivor per cc. at the end of fifty minutes, the rate of killed declined to approximately 99 per cent each twenty-minute period and at the end of thirty minutes, approximately 55,000 organisms remained viable.

TABLE 2.—Germicidal Efficiency of Sodium Silicate (Water Glass) Solutions Against Salmonella pullorum at 20 C.

DILUTION*	MINUTES EXPOSURE TO WATER GLASS BEFORE SUBCULTURE WAS MADE						
%	1	2	5	10	30	60	120
10	+	+	—	—	—	—	—
5	+	+	+	±	—	—	—
2.5	+	+	+	+	+	+	±
1.25	+	+	+	+	+	+	+

— No growth in subculture, indicating organisms killed.
+ Growth in subculture, indicating viable organisms present.
± Results variable in different trials and with different strains.
* These are the dilutions of the commercial water glass solutions.

Approximately thirty minutes were required for the next 99 per cent reduction in the number of survivors, the plate count being 450 at the end of sixty minutes exposure. Plates containing 0.1 and 1 cc. of the solution showed no growth after 120 minutes exposure. The medium used for plating was proteose tryptone agar to which 1 per cent lactose, brom cresol purple to tint, and sufficient citric acid had been added to give a final pH value of between 6.5 and 7.0 after the sodium silicate solution had been added. Such a medium should prevent the continued action of the sodium silicate solution upon the organisms.

TABLE 3.—Germicidal Efficiencies of Sodium Silicate (Water Glass) Solutions Against Salmonella pullorum in the Presence of Chicken Feces at 20 C.

*CULTURE IN 20% SUSPENSION OF CHICKEN FECES	20% WATER GLASS SOLUTION	WATER	EXPOSURE						
			1 MIN.	2 MIN.	5 MIN..	10 MIN.	30 MIN.	60 MIN.	24 HR.
CC.	CC.	CC.							
2.5	7.5	0	+	±	—	—	—	—	—
5	5	0	+	+	—	—	—	—	—
2.5	2.5	5	+	+	+	+	+	+	—
7.5	2.5	0	+	+	+	+	+	+	+

*Four parts 25% (W/V) suspension of chicken feces, autoclaved, and cooled and 1 part 24-hour broth culture of *S. pullorum*.

TABLE 4.—Germicidal Efficiency of Sodium Silicate (Water Glass) Solutions Against Escherichiacoli at 20 C.

Concentration of Sodium Silicate Solution %	Time in Minutes							
	Without feces				10% feces added*			
	5	10	20	30	5	10	20	30
25	..	..	..	..	±	—	—	—
20	±	—	—	—	±	±	—	—
15	±	—	—	—	±	±	—	—
10	±	±	—	—	±	±	±	—
2.5	+	+	±	±	..	..	..	..
1	+	+	+	+	..	..	..	..

*One Gm. chicken feces added to 10 cc. sodium silicate solution used in test.

The Disinfection of Contaminated Cardboard

Five Gm. of fresh chicken feces were smeared on each 100 square centimeters of cardboard. These were allowed to dry for twenty-four hours and then painted with sodium silicate solutions of various concentrations. After twenty-four hours, approximately 0.1 Gm. amounts of these feces were removed by means of a sterile spatula and transferred to fermentation tubes of lactose broth. From all tubes showing the presence of gas after twenty-four hours incubation, transfers were made into brilliant green lactose bile broth and then, if growth occurred, Levine's eosin methylene blue agar plates were streaked. As shown in table 6, concentrations approximating equal parts of water and the sodium silicate solutions as sold commercially were the most efficacious, probably because the viscosity of the less dilute preparations prevented them from coming in contact with all of the feces. Very dilute products were not sufficiently well buffered to maintain a germicidal hydroxyl ion concentration in the presence of the large amount of feces.

Similar experiments, in which feces were dried on cardboard, partially removed by scraping, the surface painted, and then the underlying cardboard removed and cultured, gave almost parallel results. When previously contaminated and treated boxes were again contaminated and treated, the results were even more favorable.

Sodium Silicate-Treated Boxes Noninjurious to Chicks.—A chick shipping box was heavily treated with sodium silicate and sent to Western Washington, where baby chicks were placed in the box, but the customary padding was omitted, thus bringing the feet of the chicks directly in contact with the treated surfaces. The chicks were shipped to Eastern Washington and kept in the box until they had spent a total of seventy-two hours. The feet were not injured.

TABLE 5.—Germicidal Efficiencies of Water Glass (Sodium Silicate) Solutions Against Staphylococcus aureus 20 C.

Dilutions* %	Minutes exposure to water glass before subculture was made					
	5 min.	10 min.	30 min.	60 min.	120 min.	24 hrs.
100	—	—	—	—	—	—
50	+	—	—	—	—	—
25	+	+	+	+	—	—
12.5	+	+	+	+	+	—
"K"						
100	—	—	—	—	—	—
50	+	+	—	—	—	—
25	+	+	+	+	+	—
12.5	+	+	+	+	+	+
"C"						
100	—	—	—	—	—	—
50	+	+	+	±	—	—
25	+	+	+	+	+	—
12.5	+	+	+	+	+	+

* These are the dilutions of the commercial sodium silicate solutions. The pH values of the solutions are given in table 1.
— No growth in subculture, indicating organisms killed.
+ Growth in subculture, indicating viable organisms present.
± Results variable in different trials and with different strains.

Effect of Solutions of Sodium Silicate on Strength of Corrugated Fiberboard.—Samples of fiberboard 22″x2″ were cut from chick shipping boxes and the inner surfaces treated with a series of dilutions of commercial brand of sodium silicate, "C." The samples were permitted to dry twenty-four hours and then tested by the technique of the transverse beam test described by

McCready and Katz.[11] This method consists of a load supplied by a saddle in the center of the sample which is supported near its ends by horizontal rods.

The treated samples displayed a marked increase in strength over untreated material, ranging from an increase of 3.5 per cent for a sample treated with a 20 per cent solution, to an increase of 44.5 per cent for samples treated with a 100 per cent solution of sodium silicate. In addition, the treated samples were more resilient and less brittle than the untreated fiberboard.

Sodium Silicate Solutions	Load at Rupture	Increase in Strength
%	Gm	%
100	935	44.5
80	842	30.3
60	840	29.9
40	713	10.2
20	670	3.5
Controls	647	..

Summary

Commercial water glass solutions (mixtures of $Na_2Si_4O_9$ with more alkaline sodium silicates), containing from 8.9 per cent Na_2O and 28.3 per cent SiO_2 to 14.5 per cent Na_2O and 29 per cent SiO_2, are effective germicides against gram-negative organisms. The phenol coefficient of these solutions is between 0.1 and 0.2, the more alkaline mixtures being more actively germicidal.

Painting baby chick shipping boxes, between each reuse and after rough scraping to remove most of the accumulated feces, with equal parts of commercial water glass solution and water is recommended where the boxes must be reused because of present shortages. When carefully done, this treatment will kill the more common poultry pathogens, except *Mycobacterium tuberculosis*, does not weaken cardboard boxes, and is not injurious to baby chicks shipped in treated boxes.

TABLE 6.—Efficiency of Sodium Silicate Solutions in Disinfecting Cardboard Boxes Contaminated with Feces.

Solution	Subcultures Negative for E. *coli*			
	Trial I*		Trial II**	
	C	N	C	N
%	%	%	%	%
100	90	100	50	75
80	100	100	87	75
60	100	100	100	75
40	100	100	100	100
20	100	100	100	100

24 hours exposure at room temperature.
*Feces removed before disinfecting.
**Feces not removed before disinfecting.

[10]Beckman: National Technical Laboratories, South Pasadena, Calif., Bul. 95, (1941).

[11]McCready, D. W., and Katz, D. L.: A Study of Corrugated Fiberboard. The Effect of Adhesive on the Strength of Corrugated Board. Engineering Research Bul. 28, (1939), pp. 5-9.

The Effect of Brucella Abortus on the Bovine Udder and Its Secretion

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SCHROEDER AND COTTON[1] and Smith and Fabyan,[2] in 1911, reported that cows infected with *Brucella abortus* may shed this organism in their milk. Henry Haring, and Traum[3] isolated *Br. abortus* from the milk in 93.6 per cent of the tests on cows having blood titers of 1 : 200 or over, and Bang and Bendixen[4] found an 85 per cent correlation between blood titers of 1 : 100 and over and the elimination of *Br. abortus* in the milk. The effect of this pathogen on the mammary gland and its secretion has received only limited attention from investigators, and, therefore, when the opportunity presented itself to study the udders of cows in a brucellosis project at this Station, the work reported in this paper was undertaken.

METHODS

The udders of 5 normal control cows and of 6 infected with *Br. abortus* were used in this study. Infection was accomplished in 1 cow through association with infected animals and in 5 cows by instillation of 0.1 cc. of a heavy suspension of virulent *Br. abortus* into each eye. The course of the infection was followed by repeated agglutination tests on blood serum and whey, by direct cultures on cooked blood agar containing 1 : 200,000 dilution of gentian violet, of dry udder contents or cream and sediment, and by the inoculation of guinea pigs with dry udder contents, colostrum, suspensions of placental and fetal tissues, and cream and sediment for the isolation of *Br. abortus*.

Tests for Mastitis.—After abortion or normal calving, the cows were milked by hand twice daily. The secretion of each quarter was tested, usually at weekly intervals, by means of the strip cup, the brom-cresol-purple pH, and the chlorine tests, and by the leucocyte count. The milked-out udder was palpated for evidence of fibrosis. Milk samples from the individual quarters were plated, in dilutions of 1 : 10 and 1 : 100, in veal infusion agar containing 5 per cent of fresh horse blood. After incubation at 37 C. for forty-eight hours, the plates were examined for streptococci, staphylococci, and other bacteria. A microscopic test for streptococci was also made on each sample using stained smears of incubated milk.

Gross and Microscopic Examination of the Mammary Tissue.—At the termination of the experiment, each cow was slaughtered and its udder was removed and preserved for study according to the method described by Schalm and Haring.[5] This consisted of filling the duct and vascular systems with 20 per cent formalin, followed by storing the entire udder in 10 per cent formalin. After fixation was complete, each quarter was sliced serially, at a thickness of 3 to 4 mm., in a mechanical meat slicer. A careful examination for gross lesions was then made and blocks of tissue were removed for microscopic study.

Hematoxylin-eosin was used routinely in staining the tissues and when histopathological changes were found, further sections were cut and special stains were employed, such as iron-hematoxylin-aniline-blue and Van Geison's stains for connective tissue, Verhoeff's stain for elastic tissue, silver-gold impregnation for reticulum, and Giemsa's, Jenner-Giemsa's, Unna-Pappenheim's, Loeffler's methylene-blue, and Mallory's phloxine-methylene-blue stains for bacteria.

COW HISTORIES

The 5 normal control cows were 137, 197, 227, 237, and 245. Cows 137, 237, and 245 were first-calf heifers, while 197 and 227 were second-calf animals. All carried their

From the Veterinary Science Division, University of California, Berkeley, in coöperation with the Bureau of Animal Industry, U. S. Department of Agriculture.

[1]Schroeder, E. C., and Cotton, W. E.: The Bacillus of Infectious Abortion Found in Milk. Twenty-eighth Ann. Rept., U. S. Dept. Agric., Bureau Anim. Indust., (1911), p. 139.

[2]Smith, T., and Fabyan, M.: Ueber die pathogene Wirkung des *Bacillus abortus* Bang. Centralbl. f. Bakt., Abt. I. Orig., Bd. *61* (1911-1912), S. 549.

[3]Henry, B. S., Haring, C. M., and Traum, J.: *Br. abortus* Shedder Condition in Twenty Cows. Hilgardia, *9* (1935), p. 545.

[4]Bang, O., and Bendixen, H. C.: Untersuchungen über latent Euterinfektionen beim Rind, hervorgerufen durch Abortusbakterien. Zeitschr. Infektionskrankh, u. Hyg. Haustiere. Bd. 42 (1932), S. 81; abstr. in Biol. Abstr., (1933), No. 16187).

[5]Schalm, O. W., and Haring, C. M.: A Method of Preparing Bovine Udders for the Study of the Pathology of Mastitis. J.A.V.M.A., *94* (1939), p. 372.

calves to term and were free from infection with pathogenic organisms. Cow 245 was dried off after nine weeks of lactation, to provide a normal nonfunctioning udder for comparison with the dry infected udder.

The 6 cows infected with *Br. abortus* were 192, 204, 210, 215, 246, and 255. Much of the data regarding the course of the infection in each cow is presented in table 1. Further history on each animal is as follows:

Cow 192.—Ten days after exposure to *Br. abortus* by conjunctival infection, the blood titer had changed from negative to a complete agglutination in a dilution of 1:400, and it remained at or above this level until the animal was slaughtered. After abortion, *Br. abortus* was isolated from the placenta and fetal tissues, as well as from the colostrum of each quarter. This animal was not milked; therefore, its udder reverted to the dry state within a few weeks.

Cow 204.—This animal was free from infection with *Br. abortus* during her first pregnancy and lactation. She was milked regularly by hand for nine months, and the secretion from the individual quarters was examined once a week by means of the various milk tests. Her udder was normal in every respect, except that the average chlorine content of the milk from each quarter exceeded 0.15 per cent, which is considered by some investigators to be the

TABLE 1.—Data on the Course of the Experimental Infections with Brucella Abortus.

Cow	No. Calves	Method[2] of Infection	No. Days of Gestation when: Exposed to *Br. abortus*	No. Days of Gestation when: Birth or Abortion occurred	No. Days Between Exposure to Infection and: First Isolation of *Br. abortus* from Udder	No. Days Between Exposure to Infection and: Slaughter	No. Days of Lactation when Slaughtered	Quarter	No. Days Between: First and Last Isolation *Br. abortus* from Udder	No. Days Between: Last Isolation of *Br. abortus* from Udder and Slaughter	Results of Trials to[3] Isolate *Br. Abortus* from Udder: G.P. +No.	G.P. −No.	G.V.C.B.A. +No.	G.V.C.B.A. −No.
								RF*	100	34	2	1	4	6
204	2	Contact	198	253	46	180	125	RR	134	2	3	0	7	3
								LF	134	2	2	1	6	4
								LR	125	2	2	1	6	4
210[1]	1	Eye	216	278	43	199	138	LR	16	138	1	3	1	7
								RF	131	27	1	2	4	7
215	1	Eye	200	245	45	205	160	RR	Colostrum only	160	1	2	1	11
								LF	158	2	1	2	5	11
								LR	158	2	3	0	2	11
								RF	Colostrum only	175	0	4	1	8
246	1	Eye	188	203	15	199	175	RR	Colostrum only	175	1	3	1	8
								LF	68	107	2	2	1	8
								LR	68	107	2	2	1	8
								LF	292	1	4	1	24	7
255	1	Eye	154	229	17	323	247	LR	292	1	5	0	21	10
								RF	324	5	2	0	9	2
192	1	Eye	212	250	21	356	Dry cow	RR	324	5	2	0	8	3
								LF	324	5	2	0	8	3
								LR	324	5	2	0	8	3

*RF = right front; RR = right rear; LF = left front; LR = left rear.
[1]Vaccinated when 9 months old, using 20 cc. of culture 19.
[2]Eye = 0.1 cc. of virulent *Br. abortus* suspension dropped into each eye.
[3]G.P. = Guinea pig. G.V.C.B.A. = 1:200,000 dilution of gentian violet in cooked blood agar.

maximum level for normal milk. During her second pregnancy, she was placed in contact with cows infected with *Br. abortus*. On the twenty-seventh day of contact exposure, her blood titer was 1 : 50; from the thirty-first to the forty-eighth day, it was 1 : 200, and thereafter it was 1 : 400 or above until the animal was slaughtered. After abortion, *Br. abortus* was recovered from the placenta and from the colostrum of each quarter.

TABLE 2.—Reactions to the Various Milk Tests of Milk Tests from Quarters Infected with Brucella Abortus.

Cow	No. Calves	No. Times Milk Tested	Month of Lactation when Slaughtered	Quarter	Strip Cup[1]	BCP[1] pH Test	Percentage of Chlorine			Leucocyte Count (000 Dropped)			Average Bacterial Count
							Min.	Max.	Aver.	Min.	Max.	Aver.	
204	2	17	Fifth	RF*	N	N	0.142	0.199	0.168	15	125	75	1,130
				RR	N	N	0.143	0.191	0.165	15	67	44	230
				LF	N	N	0.142	0.188	0.168	22	232	69	180
				LR	N	N	0.141	0.191	0.169	22	60	36	700
210	1	10	Fifth	LR	N	N	0.089	0.116	0.102	30	90	54	110
215	1	11	Sixth	RF	N	N	0.091	0.145	0.113	7	105	38	70
				RR	N	N	0.092	0.138	0.113	22	97	48	50
				LF	N	N	0.091	0.145	0.109	15	45	30	110
				LR	N	N	0.088	0.132	0.109	7	82	34	90
246	1	12	Sixth	RF	N	N	0.075	0.110	0.090	22	97	62	50
				RR	N	N	0.077	0.106	0.098	22	150	67	40
				LF	N	N	0.072	0.116	0.090	30	82	56	30
				LR	N	N	0.077	0.110	0.089	15	127	45	90
255	1	24	Eighth	LF	N	N	0.117	0.167	0.143	22	330	147	560
				LR	N	N	0.119	0.180	0.147	30	210	91	360

*RF = right front; RR = right rear; LF = left front; LR = left rear.
[1]N = Normal.

Cow 210—When 9 months old, this animal was vaccinated, subcutaneously, with 20 cc. of *Br. abortus* culture 19. Twenty-three months later, or on the 216th day of gestation, it was exposed to a heavy infection with virulent *Br. abortus*. It gave birth to a weak calf on the 278th day of pregnancy. From the eleventh to the thirty-third day after exposure to infection, the blood titer was 1 : 25; from the thirty-seventh to the fifty-seventh day, it was 1 : 50; on the sixty-fourth day, it was 1 : 100, and after the eighty-fourth day and until slaughter it was 1 : 400 or over. The placenta was not found, but *Br. abortus* was recovered from vaginal swabs and from the colostrum of the left rear quarter. It was not isolated again from this quarter.

Cow 215.—On the eleventh day after exposure to *Br. abortus*, the blood titer was 1 : 25; from the fifteenth to the thirty-sixth day, it was 1 : 100, and thereafter it fluctuated between 1 : 200 and 1 : 400 until the animal was slaughtered. After abortion, *Br. abortus* was recovered from the placenta, fetal tissues, and from the colostrum of each quarter.

Cow 246.—On the eighth day after exposure to *Br. abortus*, the blood titer of this animal was 1 : 50; on the eleventh day, it was 1 : 200; and from the fifteenth day to slaughter, it was 1 : 400 or above. *Br. abortus* was recovered from the placenta, the fetal tissues, and from the colostrum of each quarter.

Cow 255.—On the eighth day after exposure to *Br. abortus*, the blood titer showed a partial agglutination in 1 : 25 and a trace in 1 : 50. From the eleventh day

until the animal was slaughtered, however, it was 1 : 400 or above. When aborted, the fetus was enclosed in the placenta, and it was apparent that it had been dead for some time. *Br. abortus* was recovered from the placenta, the fetal tissues, and from the colostrum of each quarter. All quarters remained infected with *Br. abortus* throughout the period of study; but, in the third month of lactation, the right front and right rear quarters became infected with staphylococci, which caused a mild clinical mastitis. To avoid confusion, the data on these two quarters have not been included in the tables.

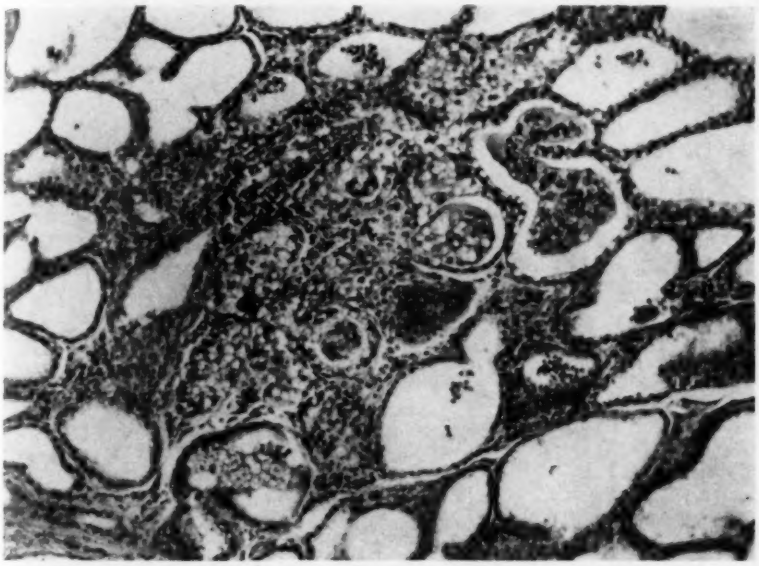

Fig. 1.—Fatty degeneration of the alveolar epithelium and exudation of neutrophiles into the alveolar lumen. Right front quarter of cow 204. x72.

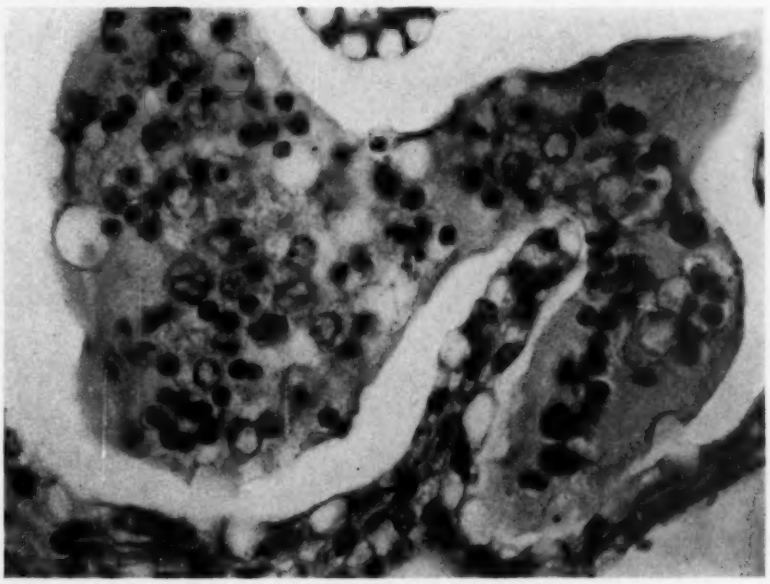

Fig. 2.—Same lesion as fig. 1, showing exudate in an alveolar lumen. Note pycnotic neutrophiles and desquamated epithelial cells. x350.

The Effect of Br. abortus Infection in the Udder on the Quality of the Milk.—The milk from the udders infected with *Br. abortus* was examined, usually at weekly intervals, by means of the various

tests previously mentioned. To provide a basis for comparison, identical analyses were made on the milk from 6 normal udders. The results are presented in tables 2 and 3.

From the tables, it can be seen that normal milk and that from the *Br. abortus* infected quarters did not differ significantly. In obtaining an average of all tests on both the normal and the infected quarters, the chlorine content of infected milk was found to average 0.125 per cent, as compared with 0.133 per cent for the normal product; the average number of leucocytes per cc. of infected milk was 53,000, as compared with 82,000 in the normal milk, and the average count of udder saprophytes was 250 per cc. in the milk from *Br. abortus* infected quarters, as compared with 640 per cc. in the normal product.

Cow 204 was among the normal animals during her first lactation and in the infected group during her second period. A comparison of her data before and after infection with *Br. abortus* shows that no significant changes took place in the quality of the milk as a result of the infection.

Palpation of the udders for indurations did not reveal any significant differences between normal quarters and those infected with *Br. abortus*. The udder of cow 204 appeared to be sensitive to deep palpation when infected with *Br. abortus*, a condition which was not noticed during her first lactation period when not infected.

TABLE 3.—Reaction to the Various Milk Tests of Milk From Normal Udders.

Cow	No. Calves	No. Times Milk Tested	Month of Lactation when Slaughtered	Quarter	Strip Cup[2]	BCP[3] pH Test	Percentage of Chlorine			Leucocyte Count (000 Dropped)			Average Bacterial Count
							Min.	Max.	Aver.	Min.	Max.	Aver.	
137	1	7	Third	RF*	N	N	0.132	0.153	0.142	60	120	67	130
				RR	N	N	0.122	0.149	0.136	22	75	50	250
				LF	N	N	0.125	0.157	0.140	15	67	37	100
				LR	N	N	0.131	0.155	0.143	30	82	50	200
197	2	7	Third	RF	N	N	0.101	0.123	0.114	15	345	114	1,400
				RR	N	N	0.110	0.129	0.116	7	52	28	100
				LF	N	N	0.108	0.136	0.116	7	35	24	800
				LR	N	N	0.105	0.137	0.114	22	37	26	100
204	1	33	—[1]	RF	N	N	0.146	0.193	0.164	0	315	68	720
				RR	N	N	0.141	0.184	0.162	0	180	46	760
				LF	N	N	0.143	0.185	0.161	0	285	45	775
				LR	N	N	0.149	0.186	0.167	0	247	57	150
227	2	26	Sixth	RF	N	N	0.118	0.157	0.136	0	405	140	900
				RR	N	N	0.113	0.150	0.137	0	420	138	1,700
				LF	N	N	0.090	0.150	0.136	0	270	114	1,200
				LR	N	N	0.114	0.150	0.136	0	450	136	1,300
237	1	28	Seventh	RF	N	N	0.122	0.156	0.137	15	225	145	580
				RR	N	N	0.123	0.152	0.135	15	210	130	390
				LF	N	N	0.121	0.151	0.132	90	345	145	750
				LR	N	N	0.120	0.162	0.139	0	405	152	1,060
245	1	9	Dry[2]	RF	N	N	0.080	0.123	0.105	22	169	95	1,600
				RR	N	N	0.089	0.145	0.118	37	127	67	120
				LF	N	N	0.087	0.124	0.106	15	32	20	100
				LR	N	N	0.088	0.120	0.108	22	120	70	250

*RF = right front; RR = right rear; LF = left front; LR = left rear.
[1]Cow 204 kept for infection with *Br. abortus* during second pregnancy.
[2]Cow 245 dried off after nine weeks of lactation; slaughtered three months later.
[3]N = Normal.

Hofstad,[6] in a study on 6 cows, arrived at the conclusion that *Br. abortus* is capable of altering the secretion of infected udders, but he found that when the number of organisms shed in the milk was low, changes in the secretion were slight or absent. Bryan and Meyer[7] found that *Br. abortus* may cause a low grade mastitis, but the milk from the majority of the infected cows studied compared favorably with the normal product. Prouty[8] discovered no significant differences in the leucocyte count of milk from *Br. abortus* infected and normal quarters, the average being 145,000 and 185,000 per cc, respectively. Both Tweed[9] and Cooledge,[10] how-

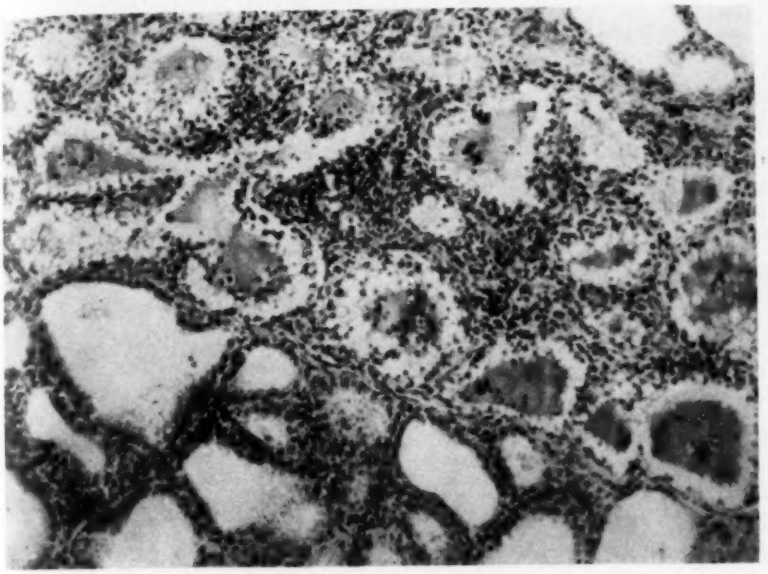

Fig. 3.—Fatty degeneration of the alveolar epithelium and exudation of neutrophiles into both the alveolar lumen and adjacent interstitial tissue. Right front quarter of cow 204. x72.

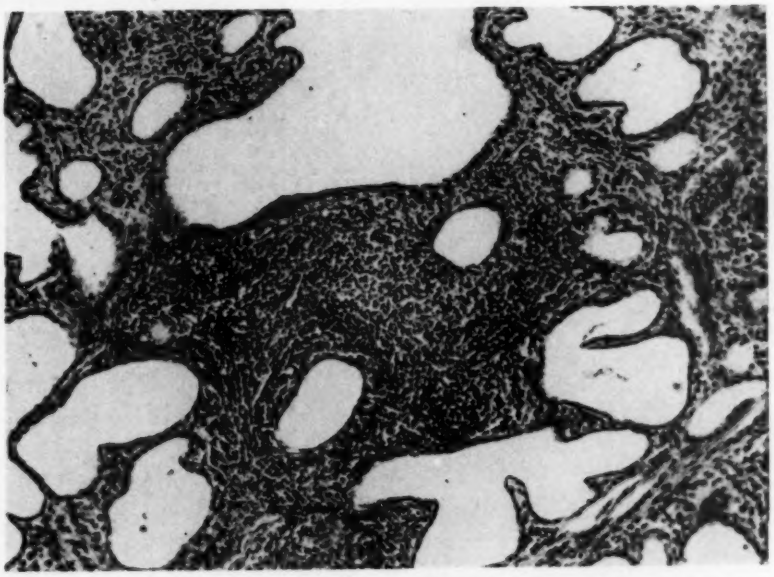

Fig. 4.—Interstitial lesion, showing a dense infiltration of lymphocytes and plasma cells. Left rear quarter of cow 255. x72.

[6]Hofstad, M. S.: The Changes Produced by *Br. abortus* in the Milk and Udder of Cows Infected with Bang's Disease. Cornell Vet., *32* (1942), p. 289.

[7]Bryan, C. S., and Meyer, D. B.: The Effect of *Brucella abortus* Infection of the Udder on the Quality of Milk. J.A.V.M.A., *89* (1936), p. 199.

[8]Prouty, C. C.: Studies on the Leucocyte Content of Milk Drawn from *Br. abortus* Infected Udders. J. Bact., *27* (1934), p. 293.

ever, reported that the leucocyte count of milk infected with *Br. abortus* was considerably higher than that of normal milk. Minett and Martin,[11] in a study of 193 lactations in 130 Holstein-Fresians, observed a reduction in corrected milk yield of 20.7 per cent, which they attributed to *Br. abortus* infection.

Gross and Microscopic Pathology in Br. abortus Infected Udders.—The method used in this study of preparing serial slices of the entire udder made it possible to examine every portion of the parenchyma for macroscopic lesions. A careful study of both normal and infected quarters failed to reveal differences which could be interpreted as gross lesions produced by *Br. abortus*. Giltner, Huddleson, and Tweed[12] expressed the view that, since the number of *Br. abortus* organisms in milk is small, the possibility of finding lesions is remote, and they cited the work of Friedemann,[13] who found neither gross nor microscopic lesions in the udder of 1 infected cow. Runnels and Huddleson[14] and Schroeder[15] also failed to find macroscopic lesions in *Br. abortus* infected udders, while Ridala[16] and Pedersen and Bass[17] observed what they considered to be gross lesions of brucellosis in the udder.

Since macroscopic changes were not found by us, blocks of tissue, approximately 1 cm. square, were removed from each quarter for sectioning according to a uniform plan. The serial slices were segregated into groups representing the anterior, middle, and posterior thirds of the quarter and, from each group, blocks of tissue were taken from the ventral, middle, and dorsal portions of the parenchyma. The tissues were prepared for sectioning in the usual manner and stained as previously described.

Definite histopathological changes were found in 17 of the 19 *Br. abortus* infected quarters. The 2 quarters that failed to show microscopic lesions had shed *Br. abortus* in the colostrum only, 175 days prior to slaughter of the animal. Hallmann, Sholl, and Delez,[18] Sholl and Torry,[19] Sholl,[20] Pedersen and Bass,[17] Runnels and Huddleson,[14] Ridala[16] and Hofstadt[6] have described histopathological changes in *Br. abortus* infected udders.

The microscopic changes observed in the *Br. abortus* infected quarters in this study were focal in character and were found primarily in the interstitial tissues, although foci affecting the alveoli were the principal lesions in the right front, right rear, and left front quarters of cow 204 and the left rear quarter of cow 210.

The alveolar lesion consisted of an exudation into the lumen of neutrophiles, some of which were in various stages of pycnosis, and a definite series could be established to follow their disintegration (fig. 1 and 2). Often the epithelium contained an excessive amount of fat and showed a tendency toward desquamation so that some epithelial cells occurred in the exudate. A local hyperemia was seen at times and infrequently extravasated erythrocytes appeared in the alveolar lumen; the adjacent interstitial tissue occasionally showed a variable infiltration by neutrophiles (fig. 3).

In the majority of the *Br. abortus* in-

[9]Tweed, R. L.: Relation of High Cellular Counts to *Bact. abortus* Infection of the Udders. Mich. Agr. Exp. Sta., Tech. Bul. 61, (1923).

[10]Cooledge, L. H.: Studies upon the Bacterial Flora of Milk with High Cellular Counts as Drawn from Apparently Normal Udders. Mich. Agr. Expt. Sta., Tech. Bul. 41, (1918).

[11]Minett, F. C., and Martin, J. W.: The Influence of Mastitis and *Br. abortus* Infection upon the Milk Yield of Cows. J. Dairy Res., 7 (1936), p. 122.

[12]Giltner, W., Huddleson, I. F., and Tweed, R. L.: The Rôle of the Udder and its Secretion in Bovine Infectious Abortion. J.A.V.M.A., *62* (1922), p. 172.

[13]Friedemann, O. H.: A Study of a *Bact. abortus* Infected Udder. Thesis, Mich. Agr. College, (1922).

[14]Runnels, R. A., and Huddleson, I. F.: The Nature of *Bact. abortus* Infection in the Udder of the Bovine. Cornell Vet., *15* (1925), p. 376.

[15]Schoeder, E. C.: Milk as a Carrier of Infection. Proc. Am. Assoc. Med. Milk Comm., (1911), p. 131.

[16]Ridala, V.: Inquiries into the Pathogenic Effects Produced by *Br. abortus* in the Udder and Certain Other Organs of the Cow. Universitatis Tartuensis (Dorpatensis), Acta el Commentationes, *31* (1937), p. 1.

[17]Pedersen, H. O., and Bass, E.: Histologische Untersuchungen über das normale und über das infizierte Euter von Färsen. Münch. tier. Wchnschr., Bd. *86* (1935), S. 301.

[18]Hallman, E. T., Sholl, L. B., and Delez, A. L.: Observations on the Pathology of *Bacterium abortus* Infections. Mich. Agr. Exp. Sta., Tech. Bul. 93, (1928).

[19]Sholl, L. B., and Torry, J. P.: Contribution to the Bacteriology and Pathology of the Bovine Udder. Mich. Agr. Exp. Sta. Tech. Bul. 110, (1931).

[20]Sholl, L. B.: Some Observations on the Pathology of *Br. abortus* Infected Udders of Heifers. Cornell Vet., *25* (1935), p. 354.

fected quarters, the most common change was an exudation of neutrophiles, lymphocytes, and plasma cells into the subepithelial and interalveolar tissues. The neutrophiles predominated but rarely, for, as a rule, the inflammatory focus consisted of a dense accumulation of lymphocytes and plasma cells (fig. 4). In a few instances, the exuded cells were observed to be filtering between the epithelial cells lining an alveolus, and when this condition became pronounced, erosion of the epithelium resulted. Usually, however, the inflammatory process remained confined to the interstitial tissues and there was no evidence of injury to the epithelium. Proliferation of the supporting connective tissue elements occurred in the larger foci (fig. 5). Epithelioid and also giant cells were found in some of the interstitial lesions in the left rear quarter of cow 215, in the left front and left rear quarters of cow 255, and in the right rear,

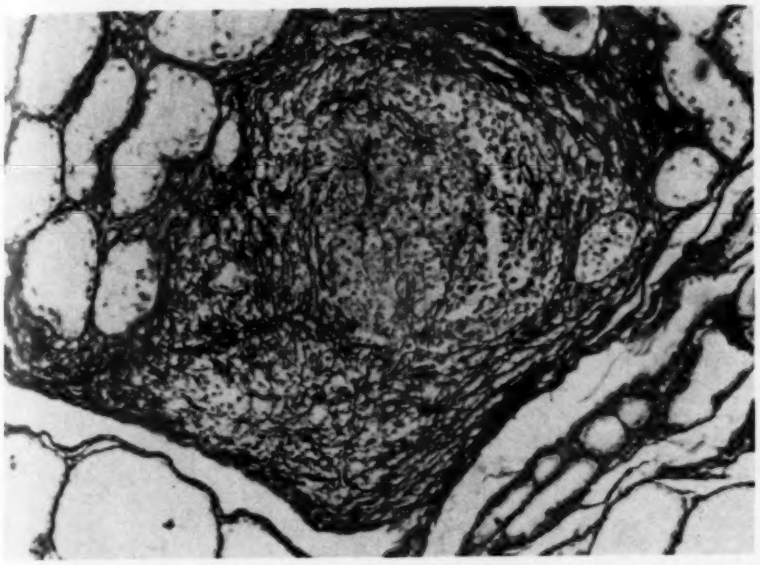

Fig. 5. — Interstitial lesion, showing an increase in reticulum fibers in the inflammatory focus. Left rear quarter of cow 204. x72.

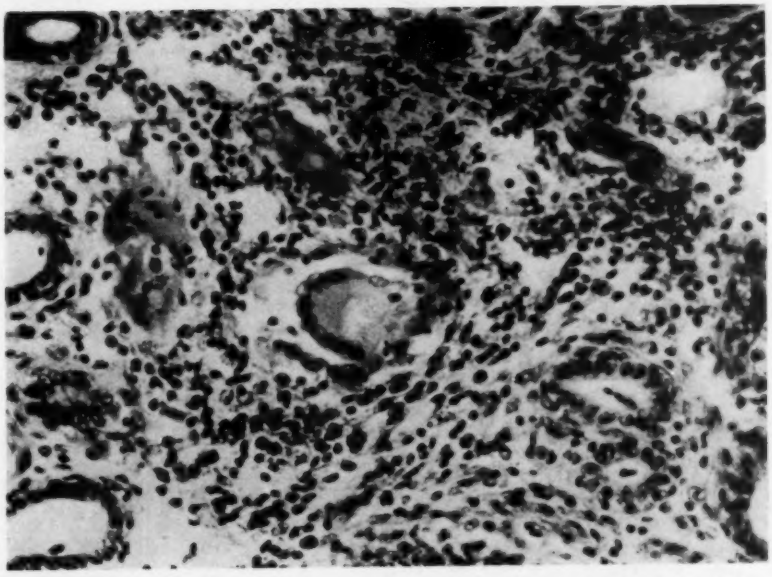

Fig. 6. — Interstitial lesion, showing lymphocytes, plasma cells, epithelioid cells, and a giant cell. Right rear quarter of cow 192. x180.

left front, and left rear quarters of cow 192 (fig. 6). Jaffé[21] has stated that the histopathological changes in guinea pigs experimentally infected with *Br. abortus* and *Brucella melitensis* are similar to those of experimental tuberculosis, and he reported finding giant cells in the spleen lesions.

Approximately, 70 per cent of the section from the *Br. abortus* infected quarters contained microscopic changes, and they occurred in all portions of the parenchyma. rather than to the actual occurrence of a greater number of lesions in the dry gland. The damage to the secretory tissue was relatively small, which perhaps explains why the milk was not abnormal.

Histopathological changes were also observed in the supramammary lymph nodes of some of the infected udders. They were characterized by a variable degree of infiltration of neutrophiles into both the cortex and the medulla, which was sometimes

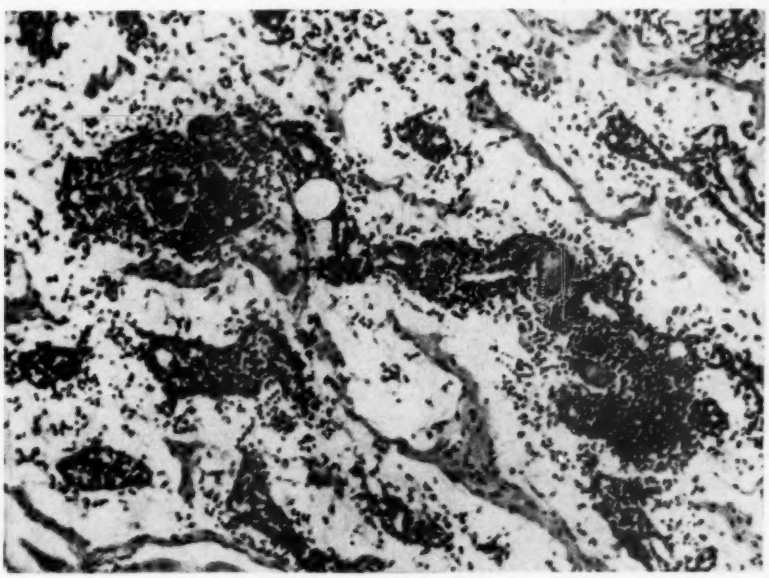

Fig. 7.—Left supramammary lymph node, showing three giant cells in the medullary cords. Cow 215. ×72.

Each inflammatory focus was, as a rule, limited to the involvement of a few alveoli, although infrequently a more extensive area was affected. The inflammatory foci were usually so widely separated that more than two lesions were seldom observed in a single section of 1 cm.^{2} of parenchyma. These foci were found with greater frequency in the dry quarters than in the lactating, which probably can be attributed to the concentration of the interstitial tissue (which contains most of the inflammatory foci) during involution of the parenchyma associated with hyperemia and hemorrhage. In some sections, epithelioid cells and giant cells were found (fig. 7).

A special effort was made to stain *Br. abortus* in the tissues. Faintly stained bodies resembling this organism were seen in some of the inflammatory foci when Giemsa's and Mallory's phloxine-methylene-blue stains were used. These bodies did not stain intensely and thus could not be photographed. Similar structures were not found in the sections of normal udder tissue.

SUMMARY

The quality of the milk from *Brucella abortus* infected quarters was not significantly different from the normal product.

[21] Jaffé, R. H.: Über die experimentelle Infektion des Meerschweinchen mit dem Bacillus melitensis dem Bacillus abortus. Virchow's Arch., Bd. *238* (1922), S. 119. (Cited by Poppe, K.: Kolle und Wassermann: Handbuch der pathogenen Mikroörganismen, 3rd ed., *6* (1929), p. 693.)

Visible particles were not observed in the foremilk, and the pH, chloride content, leucocyte count, and saprophytic bacterial count were well within normal limits.

A careful examination of the entire parenchyma of each infected quarter failed to reveal gross lesions. Histopathological changes were observed, however, in 17 out of 19 quarters that had harbored *Br. abortus* for periods varying from sixteen to 324 days. These consisted of an exudation of neutrophiles into the alveolar lumen, associated with fatty degeneration of the alveolar epithelium with a tendency toward desquamation of epithelial cells. More commonly, however, the microscopic pathology was characterized by a dense accumulation of lymphocytes and plasma cells in the interalveolar tissues. Epithelioid cells and giant cells were found in some instances. In the larger inflammatory foci, proliferation of the connective tissue elements occurred. In some instances, the supramammary lymph nodes also contained histopathological changes.

Demonstration of Serological Types Within the Nonhemolytic Pasteurella

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FOR EFFECTIVE treatment of Pasteurella infections with commercial antigens and antiserums, there should be included in the immunizing mixtures all of the strains of Pasteurella which are required to cover the known antigenic variations of the species.

The existence of distinct serological types of Pasteurella has been demonstrated by many workers. In 1929, Cornelius[1] differentiated 4 types by agglutinin-absorption tests. In 1935, Yusef[2] re-examined the same strains by use of the precipitin test and confirmed 3 of these types. The strains classified by Cornelius as type 4 were placed in one of the other types.

Khalifa,[3] working independently, observed 3 types by fermentative tests and demonstrated the specificity of each type by active and passive immunization of rabbits. Khalifa was the first investigator to correlate the serological results with the fermentation of xylose, arabinose, and mannitol. Rosenbusch and Merchant[4] demonstrated 3 types of nonhemolytic Pasteurella and a hemolytic group by agglutinin tests and by xylose, arabinose, and dulcitol fermentation.

In this laboratory, the existence of 3 serologically distinct types within the nonhemolytic Pasteurella has been confirmed by passive immunization of mice and by rapid slide agglutinative tests with specific antiserums. The identity of 2 of our types (types 1 and 2) with those described by Rosenbusch and Merchant was established by use of strains previously differentiated by these authors. Our type 3 strains have not been directly identified with those classified as such by Rosenbusch and Merchant because of the unavailability of their strains. As an aid to other investigators, we have identified American Type Culture Collection strains as follows:

Type 1:	*P. avicida* 7707
Type 2:	*P. bubalseptica* 6530
Type 3:	*P. muricida* 6535

EXPERIMENTAL RESULTS OF AGGLUTINATIVE TYPING

Of the total of 30 strains of nonhemolytic Pasteurella which we have studied, 12 were classified as type 1, 11 as type 2, and 7 as type 3. The origin of the type 1 strains was as follows: avian (4), bovine (1), equine (4), and porcine (3). All of these type 1 strains were found to be morphologically similar to the "Coccobacillus" of Gamaleia.

Eight strains of *Pasteurella bubalseptica* have been identified by us as belonging to type 2. They include the United States Bureau of Animal Industry strains H-1054 and M-1226, the New York State Veterinary College strains 864 and 865, and the American Type Culture Collection strains 6530 and 7960. All of these strains appear to have originated from a herd of bison that died of acute disease in the Yellowstone National Park, in March, 1922. Their unusual virulence and antigenicity are described by Gochenour.[5] Gochenour reported that the bison strain was serologically identical with the strains of bovine origin

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[1]Cornelius, J. T.: An Investigation of the Serological Relationships of 26 Strains of Pasteurella. J. Path. and Bact., *32* (1929), pp. 355-364.

[2]Yusef, H. S.: Serological Classification of Pasteurella Strains. J. Path. and Bact., *41* (1935), pp. 203-206.

[3]Khalifa, I. A. B.: Pasteurella in Animals and their Interclassification. Minist. Agric., Egypt, Bul. 147 (1934), 36 pp.; abstract in Vet. Bul., *6* (1936), p. 792.

[4]Rosenbusch, C. T. and Merchant, I. A.: A Study of the Hemorrhagic Septicemia Pasteurella. J. Bact., *37* (1939), pp. 69-89.

[5]Gochenour, W. S.: The Development of a Potent Immunizing Agent (Natural Aggressin by the use of Highly Virulent Strains of Hemorrhagic Septicemia Organisms. J.A.V.M.A., *65* (1924), 433-441.

which he studied. We have found only 3 other type 2 strains. These were of avian (1), bovine (1), and rabbit (1) origin. All of our type 2 strains can be readily differentiated from types 1 and 3 on the basis of morphology. In our experience, these organisms never assume the "Coccobacillus" form. They are discrete rods, from two to four times as long as the type 1 and type 3 Pasteurella.

Our type 3 strains were found to be morphologically similar to our type 1 strains. Their origin was: avian (1), bovine (1), canine (2), ovine (1), porcine (1), and mouse (1).

The type differentiation was carried out by use of rapid slide agglutinative tests with monovalent antiserums. Before testing, daily transfers were made on serum agar. This procedure was found necessary to assure maximum agglutinative specificity on the part of the organisms. Overnight growth was suspended in 1 cc. of physiological saline at the time of testing. A platinum loop 5 mm. in diameter was used to measure and transfer the culture suspension and a loop 1 mm. in diameter was used for the serum. Glass slides were flamed to remove surface film, cooled, and ruled into three compartments an inch square. Mixtures of bacterial suspension were made with each of the 3 monovalent antiserums and tested simultaneously.

The monovalent antiserums used were prepared in rabbits by intravenous injection of formolized suspensions having a density of around ten billion bacteria per cc. Doses increasing from 0.25 cc. to 2 cc. were given in a series of 3 daily injections spaced a week apart over a period of six weeks. The immunizing suspensions were prepared from eight-hour broth cultures containing in each instance ten million lethal doses per cc. for 18- to 20-Gm. mice.

The variations in agglutinative specificity which were observed among strains belonging to the same type appeared to be correlated with wide variations in virulence. All of the highly virulent strains, representing each type, failed to give cross-agglutinative reactions with the antiserums for the heterologous types. Strains of low virulence for mice were found to vary in degree of agglutinability with heterologous antiserums, but to improve after repeated subculture on serum agar. These variations in virulence and agglutinative specificity seem attributable to previous contact by the organism with specific immune substances. It is well known that serum-sensitization causes loss of virulence in the pneumococci. Opportunities for serum-sensitization may occur through the use of mediums enriched with blood or serum containing demonstrable agglutinins for the organisms cultured. One hundred and seventy-three samples of normal horse serum were examined for agglutinins for type 1 Pasteurella. Agglutinins were demonstrated in 24 per cent of the serums tested. For over a year, our collection of Pasteurella has been regularly maintained on agar enriched with horse blood or serum which has been found to be normal by the agglutinative test. None of the cultures treated in this manner have shown loss of virulence.

EXPERIMENTAL RESULTS OF PASSIVE IMMUNIZATION

The highly virulent type 1, 2, and 3 strains of Pasteurella used in the passive immunization experiments were compared with respect to degree of virulence for mice. All of these strains have been kept on ordinary laboratory mediums enriched with tested, normal horse blood or serum, and have received occasional mouse passage. Five-hour beef heart broth cultures were used. Three mice were used on each dilution. Twenty-eight virulence titrations were performed on 2 type 1 strains; 56 virulence titrations were performed on 2 type 2 strains; and 7 virulence titrations were performed on 1 type 3 strain. It was regularly found that such broth cultures of all strains contained at least ten million lethal doses per cc.

The specificity of each of the 3 serological types was demonstrated by mouse protective tests against at least ten thousand lethal doses of the above virulent cultures. From 0.5 cc. to 0.02 cc. amounts of serum were used. Simultaneous injections of culture and serum were made into the

peritoneums of the mice, which were observed for seven days.

The monovalent types 1, 2, and 3 rabbit antiserums, previously described, protected mice against death from organisms belonging to the homologous type, but failed to protect against organisms of heterologus types.

In view of our observations regarding the occurrence of natural agglutinins for Pasteurella in some samples of normal horse serum, it was expected that the same high degree of protective specificity would not be found in all samples of immune horse and cow serum. Serums from 3 groups of animals were available for study, as follows: *(a)* horses immunized with a single strain of type 1 Pasteurella, *(b)* horses and cows immunized with a strain of type 1 and a strain of type 2 Pasteurella, and *(c)* horses immunized with a strain of type 3 Pasteurella.

In testing bleedings from these animals over a period of six months, we have found as in *(a)*, *(b)*, and *(c)*, consistently fail to afford appreciable protection against the heterologous types as compared with the protection afforded against the homologous type. Heterologous protection was observed in serums from some horses and not in serums from other horses on the same treatment. This is the result which one might expect to encounter when immunizing animals which are subject to infection through natural channels with one or more of the bacterial species being studied. In no instance was the degree of heterologous protection sufficient to suggest the possibility of using a single strain for the production of a trivalent antiserum. Mixtures of serums from horses immunized with the types 1, 2, and 3 strains were the only serums which would consistently protect mice against all of the virulent, nonhemolytic Pasteurella studied.

In view of these facts, is it not illogical to select strains of Pasteurella on the basis of host origin when preparing bacterins and antiserums for the treatment of Pasteurella infections? Rather, should not these products be made trivalent in effective dosage for each of the 3 types?

SUMMARY AND CONCLUSIONS

Serological studies of 30 strains of nonhemolytic Pasteurella demonstrated that these organisms could be definitely classified into 3 distinct types. The type specificity, virulence, and host origin of these strains were found to be unrelated. The protective property of therapeutic antipasteurella serums was found to be correlated with the type specificity of the strains included in the immunizing mixtures. Antipasteurella serums representing 3 serological types were the only serums which would consistently protect mice against all of the virulent, nonhemolytic Pasteurella studied.